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**Detection and isolation of human  
pathogenic Non-O157 Shiga toxin-  
producing *Escherichia coli* (STEC) in food: A  
needle in a haystack.**

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## List of abbreviations

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A/E	attaching and effacing
AAF	aggregative adherence fimbriae
AE-STEC	eae-positive STEC
aw	water activity
bfp	bundle-forming pili
BHIB	brain heart infusion broth
BPW	buffered peptone water
CDC	centre for disease prevention and control
CFU	colony-forming units
Chr ID	chromID™ EHEC
Chr ST	CHROMagar™ STEC
CRISPR	clustered, regulatory interspaced, short palindromic repeats
Ct	qPCR cycle threshold
DAEC	diffusely adherent E coli
ddPCR	<i>droplet digital PCR</i>
ddPCR	droplet digital PCR
<i>eae</i>	<i>E. coli</i> attachment and effacing
EAF	EPEC adherence factor
EAggEC	enteroaggregative <i>E. coli</i>
EAHEC	enteroaggregative hemorrhagic <i>E. coli</i>
EAST	<i>E. coli</i> ST enterotoxin
EC	<i>E. coli</i> broth
ECDC	European centre for disease prevention and control
EEB	EHEC enrichment broth
EHEC	enterohaemorrhagic <i>E. coli</i>
EIEC	enteroinvasive <i>E. coli</i>
EMM	TaqMan® environmental master mix 2.0
EPEC	enteropathogenic <i>E. coli</i>
ER	endoplasmic reticulum
ETEC	enterotoxigenic <i>E. coli</i>
Gb3	globotriaosylceramide-3 receptor
Gb4	globotetraosylceramide receptor
gDNA	genomic DNA
GUD	β-glucuronidase
HC	hemorrhagic colitis
HUS	hemolytic-uremic syndrome
IAC	internal amplification control
ISO	international organization for standardization
LAMP	loop-mediated isothermal amplification
LEE	locus of enterocyte effacement

LOQ	limit of quantification
lpf	long polar fimbriae
LT	heat-labile
MLST	multilocus sequence typing
mMac	modified macConkey agar (Possé <i>et al.</i> , 2008)
mPCR	multiplex polymerase-chain reaction
MRA	molecular risk assessment
MUG	4-methylumbelliferyl- $\beta$ -D-glucuronide
NLE	non-LEE-encoded effector
NM	nonmotile
OI	O-islands
pAA	aggregative adherence plasmid
PAI	pathogenicity islands
PCR	polymerase-chain reaction
PFGE	pulsed-field gel electrophoresis
qPCR	real-time quantitative PCR
RAJ	recto-anal junction
RAMS	recto-anal-mucosal swab
RB	Rainbow® Agar O157
RE	Rapid <i>E. coli</i> O157:H7
SEB	Stec Enrichment Broth
ShET1	<i>Shigella</i> enterotoxin 1
SMAC	sorbitol MacConkey agar
ST	heat-stable
STEC	Shiga toxin-producing <i>E. coli</i>
Stx	Shiga toxin
T3SS	type III secretion system
TBX	tryptone bile X-glucuronide
tir	translocated intimin receptor
TSA	tryptone soy agar
TSB	tryptone soy broth
UMM	TaqMan® Universal PCR Master Mix
UNG	Uracil-DNA Glycosylase
VT	vero cytotoxins
VTEC	verocytotoxin-producing <i>E. coli</i>
X-Gal	5-bromo-4-chloro-3-iodo- $\beta$ -D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indoxyl- $\beta$ -D-glucuronide

## Chapter 1: General Introduction

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## **1. *Escherichia coli: a thin line between commensalism and pathogenicity***

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### **1.1. *Escherichia coli***

In 1885 the 27-year-old German pediatrician Theodor Escherich isolated a common colon bacterium from stool samples of healthy infants. Based on the recently described Gram staining technique and anaerobic culture methods he described a Gram-negative, facultative anaerobic bacillus and named it *Bacterium coli commune* (Escherich, 1885). This bacterium was later renamed in honor of its discoverer to *Escherichia coli*. Since the discovery of *E. coli* occurred only a decade after Robert Koch described the association between bacteria and diseases and postulated that one bacterial species was either pathogenic or not, *E. coli* was classified as a commensal bacterial species (Mainil, 2013). *E. coli* usually colonizes the human gastrointestinal tract within the first hours after birth and both human and bacterium coexists to mutual benefit. However, when the gastrointestinal barriers are violated or the hosts' immunity is insufficient these commensal *E. coli* can cause illness (Nataro and Kaper, 1998).

Since its discovery, *E. coli* was found in a broad variety of niches, either within organisms or outside in the environment. In order to survive, the bacterium has adapted to its respective biotopes and some of these highly adapted *E. coli* have acquired very specific virulence factors. These virulence factors gave *E. coli* the capability to cause disease in healthy humans. While most of the virulence factors were encoded on mobile genetic elements, the most favorable virulence combinations evolved to become “locked” permanently into the genome (Kaper *et al.*, 2004). The most important “locked” virulence factors associated with human disease were used to categorize the pathogenic *E. coli* into several specific pathotypes. These pathotypes can be grouped together based on the clinical aspect of the disease, as extraintestinal pathogenic *E. coli* (most often causing urinary tract infections, but also sepsis and meningitis) and intestinal pathogenic *E. coli* (causing enteritis and diarrhea) (Kaper *et al.*, 2004). The latter group contains six pathotypes characterized by the specific virulence genes encoding for particular colonization mechanisms or toxin production.

### **1.2. *Pathotypes***

#### **1.2.1 Enteroinvasive *E. coli* (EIEC)**

In 1898 Kioshi Shiga first described a bacillus as causative agent for bacterial dysentery. As it seemed related to *Bacterium coli commune*, he named it *Bacillus dysenteriae*. Despite the relatedness to *E. coli*, the later renamed bacterium, *Shigella dysenteriae* type 1 remained in a separate genus because the two genera could be differentiated based on their physiological,

biochemical and clinical characteristics. While most of the *E. coli* strains (>80%) are mobile, lysine decarboxylase positive, form gas from D-glucose, are lactose positive and indol-negative, *Shigella* is non-mobile, lysine decarboxylase negative, seldom forms gas from D-glucose and is lactose negative (Van Den Beld and Reubsaet, 2012). Moreover, in contrast to *E. coli*, the early stages of the *Shigella* pathogenesis consists of the invasion of epithelial cells (Figure I-1 B). However, the clear separation between both genera was hindered in 1944 by the discovery of a hybrid *E. coli*, which was able to cause dysentery and possessed characteristics more in agreement with *Shigella*, such as lysine decarboxylase negative, non-motile, lactose negative and invasiveness. These strains are now known as enteroinvasive *E. coli* (Kaper *et al.*, 2004; Van Den Beld and Reubsaet, 2012). Both *E. coli* and *Shigella* are supposed to share common ancestry, the major event that probably gave rise to both *Shigella* and EIEC was the acquisition of the invasion plasmid (pINV) (Figure I-1 A). Therefore both organisms can be identified by the presence of the multi-copy gene *ipaH* located on both plasmid and chromosome (Venkatesan *et al.*, 1991).

### 1.2.2 Enteropathogenic *E. coli* (EPEC)

EPEC was the first member of the intestinal pathogenic *E. coli* to be described and was associated with large outbreaks of infantile diarrhea. While outbreaks due to EPEC have become rare in the industrialized countries, it is still of major importance in developing countries (Jerse *et al.*, 1990; Kaper *et al.*, 2004). Despite its importance, until 1970's these organisms could only be distinguished from other *E. coli* by serotyping, based on lipopolysaccharide detected on the bacterial surface. In 1987 a consensus was reached concerning the O-groups recognized as EPEC (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158) (Jafari *et al.*, 2012). Later on it was shown that serotypes were not pathotype specific and therefore insufficient to precisely identify EPEC. Consequently, in 1995 the definition of EPEC was modified by including its ability to cause attaching and effacing lesions (A/E), the hallmark of all EPEC. The intimate attachment of the bacteria to the intestinal epithelial cells, effacement of the enterocyte microvilli and the pedestal-like structures on which the bacteria perch, produce these typical lesions (Nataro and Kaper, 1998) (Figure I-1 B). The first gene associated with A/E lesion formation, namely the *eae* (*E. coli* attaching and effacing) gene, encodes for an outer-membrane adhesin essential for the A/E lesion formation. This gene was designated as the marker gene for the pathotype EPEC (Tzipori *et al.*, 1995) (Figure I-1 A). Besides the *eae* gene, other determinants are known to be involved in the A/E lesion formation. All these genes are all located on a large conserved chromosomal

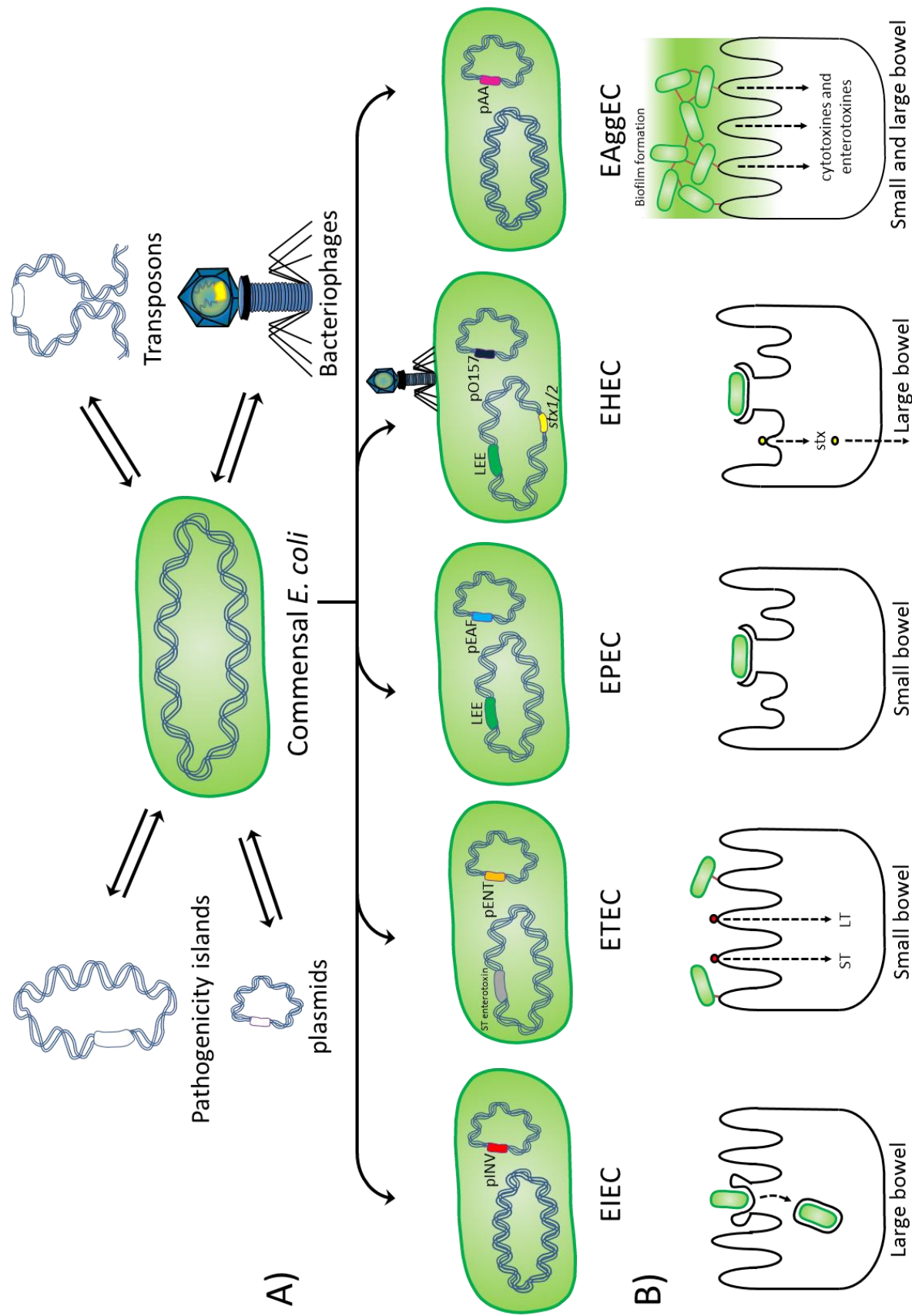


Figure I-1 A) Contribution of mobile genetic elements to the evolution of pathogenic *E. coli*. *E. coli* virulence factors can be encoded by several mobile genetic elements, including transposons, plasmids, bacteriophage and pathogenicity islands. These genetic changes can give rise to different types of pathogenic *E. coli*. B). Each pathotype of the diarrheagenic *E. coli* can interact with the enterocytes in their own unique ways. EPEC adhere to the enterocytes by inducing the characteristic A/E lesions and pedestal formation. The adhesion and A/E lesions of EPEC are encoded by the genes located on the EPEC adherence factor plasmid (pEAF) and the locus of enterocyte effacement (LEE), respectively. EHEC also induce the A/E lesion, in addition to the release of Shiga toxin (Stx) for systemic absorption. Additional adherence and virulence factors are encoded by genes located on the pO157 plasmid. ETEC adheres to the enterocytes and secretes of heat-labile (LT) and/or heat-stable (ST) enterotoxins, encoded by the genes located on the ETEC plasmid (pENT) and transposon. EAggEC adheres to enterocytes in a thick biofilm and secretes enterotoxins and cytotoxins, encoded by genes located on the aggregative adherence plasmid (pAA). EIEC invades the enterocytes and moves through the cell. The invasive factors are encoded by genes located on the invasion plasmid (pINV). Figure redrafted and adjusted from Ahmed *et al.*, 2008; Kaper *et al.*, 2004.

pathogenicity island (see 5.2 Genotyping), namely the locus of enterocyte effacement (LEE) (McDaniel *et al.*, 1995). The adherence pattern of EPEC is dependent upon the presence of a large 60 MDa EPEC adherence factor (EAF) plasmid which encodes the type IV bundle-forming pili (bfp) and several genes involved in adhesion regulation (Jafari *et al.*, 2012). Since, not all EPEC strains seem to possess this plasmid, the EPEC pathotypes were divided into typical EPEC (tEPEC) and atypical EPEC (aEPEC). The tEPEC, characterized by the presence of the *eae* gene and a large EAF plasmid, is transmitted from human to human. While, the aEPEC is a more heterogeneous group, harboring a variety of virulence factors and often present in animal reservoirs and therefore transmissible to human via contaminated food.

### 1.2.3 Enterotoxigenic *E. coli* (ETEC)

ETEC is one of the most important bacterial causes of infantile diarrhea and cholera-like disease in developing countries. Furthermore, it is one of the main causes of Travelers' diarrhea in individuals from industrialized countries of all ages visiting developing countries. ETEC was first recognized in 1968 by the discovery of an *E. coli* with the ability to produce a heat-labile (LT) enterotoxin, which resembled the toxin produced by *Vibrio cholerae*. Only later were the heat-stable (ST) enterotoxins detected. The genes encoding for these enterotoxins and the colonizing factors of ETEC are located on mobile genetic elements such as the plasmid pENT and a transposon (Yang and Wang, 2014) (Figure I-1 A). Since the infective dose is relatively high, any form of inter-human transmission is rare. The main source of ETEC infections are poor food handling hygiene and contaminated drinking water (Kaper *et al.*, 2004; Nataro and Kaper, 1998).

### 1.2.4 Enteroaggregative *E. coli* (EAggEC)

While EAggEC is the most recent discovered pathotype, at the end of the 1980s, it is considered to have been an important diarrheagenic agent since 1920. This pathogen is a major cause of diarrhea in both industrialized and developing countries and next to ETEC one of the main causes of Travelers' diarrhea. This pathotype is characterized by its peculiar diffuse adherence

pattern or biofilm to the intestinal epithelial cells, resembling the bricks of a wall (Figure I-1 B). The adhesion and biofilm formation is encoded by a gene cluster located on the aggregative adherence plasmid (pAA) and regulated by *aggR* gene, which is key in the pathogenesis and the main genetic marker for EAggEC (Figure I-1 A). In addition, several chromosomally-encoded toxins have been described for EAggEC, such as *E. coli* ST enterotoxin (EAST) and *Shigella* enterotoxin 1 (ShET1). However, their role during the pathogenesis remains unclear (Kaper *et al.*, 2004; Yang and Wang, 2014).

#### 1.2.5 Diffusely adherent *E. coli* (DAEC)

The discovery of DAEC stemmed from the research which revealed the existence of EAggEC. The observed diffuse adherence pattern to the intestinal epithelial cells could be divided into aggregative adherence, hence EAggEC and “true” diffuse adherence, hence DAEC. This pathotype represents a very heterogeneous group of *E. coli*, and while DAEC has been implicated as cause of diarrhea in several studies, both epidemiology and pathogenesis are still largely unknown (Kaper *et al.*, 2004; Tozzoli and Scheutz, 2014). The diffuse adherence has been attributed to several adhesins and invasins encoded by genes on the chromosome or plasmid (Yang and Wang, 2014).

#### 1.2.6 Shiga Toxin-producing *E. coli* – Verocytotoxin-producing *E. coli*

As mentioned above, *Shigella dysenteriae* type 1 was described as the bacterial cause of dysentery. The main virulence trait of this pathogen was the production of Shiga toxins (Stx), a toxin targeting vascular endothelium and a major contributor to the formation of bloody diarrhea (Bridgwater *et al.*, 1955; O'Brien *et al.*, 1980). Later on Shiga-related toxins were found in *E. coli* and were called Shiga-like toxin-producing *E. coli* (STEC) (Strockbine *et al.*, 1988). In the meantime, Konowalchuk *et al.* (1977) described a heat-labile toxin produced by certain *E. coli* strains with the ability to induce a cytotoxic response in Vero cells, hence Verocytotoxin-producing *E. coli* (VTEC).

### 1.3. ***Enterohaemorrhagic E. coli* (EHEC)**

The September edition of The Lancet in 1983 reported a food-borne outbreak of haemorrhagic colitis (HC) in the United States in 1982. *E. coli* O157:H7 was implied as causative agent isolated from contaminated hamburgers. This particular *E. coli* was able to produce high levels of Vero cytotoxins. Moreover, these toxins were immunologically indistinguishable from the previously described Shiga toxins. Henceforth, it could be concluded that the Shiga-like toxin and Vero cytotoxin, both produced by *E. coli*, were the same substance (O'Brien *et al.*, 1983). Because STEC

(Stx) and VTEC (Vt) indicate the same organism classification, for consistency the Stx nomenclature will be used throughout this thesis, even though both are still used in scientific publications.

Following the research concerning the outbreak during 1982, Karmali *et al.* (1983) were able to link the causative agent to a distinct clinical syndrome Hemolytic-Uremic Syndrome (HUS), which includes acute renal failure, thrombocytopenia and microangiopathy in children. Therefore, besides bloody diarrhea, the classical form of HUS is now a recognized complication of a STEC infection.

STEC is able to produce two distinct types of Shiga-like toxins, one immunologically indistinguishable from Stx produced by *Shigella dysenteriae* and another antigenetically distinct from Stx. Therefore, the following nomenclature was devised, the toxin closely related to Shiga toxin was named Shiga-like toxin 1 (Stx1) and the genetically distinct toxin was named Shiga-like toxin 2 (Stx2) (Strockbine *et al.*, 1986). Furthermore, in 1996 it was decided to omit “like” from the toxin nomenclature (Scheut *et al.*, 2012).

Besides the production of Stx1 and/or Stx2, the *E. coli* O157:H7 showed a peculiar adherence mechanism to the intestinal epithelial cells. The A/E lesions were observed, which were the hallmark for EPEC strains up until then. Moreover, the large conserved pathogenicity island LEE essential for the A/E lesion formation was detected in all of these strains (McDaniel *et al.*, 1995). The presence of LEE seemed to significantly enhance the virulence of STEC, since LEE-positive STEC strains were more often associated with severe illness compared to LEE-negative STEC strains (Navarro-Garcia, 2014). This observation led to the definition of a subgroup within STEC for those strains associated with HC, the production of one or more of the Shiga toxins, the formation of A/E lesions and the presence of a large virulence plasmid were termed “Enterohaemorrhagic *E. coli*” (EHEC). The large plasmid, also called pO157 or EHEC plasmid encodes for certain virulence and adherence factors. This dangerous cocktail of virulence factors makes sure that consumption of as few as ten STEC cells can cause human infection (Nataro and Kaper, 1998).

While originally only STEC serotypes O157:H7 and O26:H11 were classified as EHEC, the subgroup was soon extended to different serogroups (O111, O145, O45 and O4) (Karmali, 1989; Tzipori *et al.*, 1995). Up to now, over 400 STEC serotypes have been described, with more than 200 linked to human illness. *E. coli* O157:H7 remains the most common STEC serotype associated with human illness. On the other hand non-O157 STEC strains are increasingly recognized as food-borne pathogens of importance (Wang *et al.*, 2013). Still, much is unknown about the non-O157 STEC strains (Navarro-Garcia, 2014). The non-O157 STEC serogroups most frequently linked with food-

borne illnesses are the “big six” O26, O45, O103, O111, O121 and O145 for the United States and the “big five” O26, O91, O103, O111, and O145 for the European Union (EFSA, 2015; Navarro-Garcia, 2014). Karmali *et al.* (2003) developed an empirical classification scheme (seropathotypes A-E) to assess the clinical and public health risks associated with different STEC strains. This scheme is based on the reported occurrence of specific STEC serotypes and their association with outbreaks, human disease in general and HUS in particular. Seropathotype A consists of the *E. coli* O157:H7 and *E. coli* O157:NM (nonmotile), most commonly associated with outbreaks and HUS. Seropathotype B includes those non-O157 STEC strains that similarly to seropathotype A cause severe illness and outbreaks, but in lower frequencies. Seropathotype C consists of the strains linked to sporadic HUS cases but seldom with outbreaks. Both seropathotype D and E include STEC strains not associated with HC, HUS or outbreaks, isolated from humans and animals, respectively (Karmali *et al.*, 2003; Scheutz, 2014). This approach remains a valuable tool for the differentiation of human pathogenic STEC. However, not all isolated STEC strains are fully serotyped, nor is the potential virulence always known. Furthermore, as new data continually becomes available more serotypes are being included to the different seropathotypes, and therefore the definitions of the seropathotypes are somewhat flexible (Bosilevac and Koohmaraie, 2011; Buvens and Piérard, 2012).

#### **1.4. *Emerging STEC: a paradigm shift***

In May 2011 an alarming increase of HC and HUS cases was noted in Germany. Because these clinical signs are most often associated with EHEC infections, local research indicated a STEC strain belonging to serotype O104:H4 as causative agent (Piérard *et al.*, 2012). In June 2011 another outbreak caused by the same STEC O104:H4 strain was reported in France. In total more than 3 816 persons were affected, with 845 developing HUS and 54 fatalities, making this one of the largest reported STEC outbreak. In contrast with the classical form of HUS, most HUS cases were reported in adults, mostly women (EFSA, 2013a). Contaminated fenugreek seeds imported from Egypt were implicated as the source of the outbreak. Since the STEC O104:H4 strain was never actually isolated from the seeds, due to insufficient detection methods for this food matrix, some uncertainty remains. The clinical signs resembled an EHEC infection and even though the O104:H4 STEC strain produced Stx2, one of the main virulence traits of all STEC, the strain showed more similarities with another type of pathogenic *E. coli*. The particular adherence mechanism of the strain was similar to EAggEC. This in combination with Stx2 and specific additional virulence factors acquired by this O104:H4 STEC strain may explain the increased pathogenicity and the scale of this outbreak (Navarro-Garcia, 2014). Because this strain

is not able to form the typical A/E lesion, it cannot be classified as a EHEC strain, even though the media and some scientific publications continue to use the term EHEC (Piérard *et al.*, 2012).

The outbreak in 2011 and the isolated O104:H4 STEC strain caused a shift in the pathogenic STEC paradigm, which was dependent on the presence of the well-known *eae* virulence marker for EHEC. This LEE-negative, pO157-negative STEC strain was able to cause severe illness attributed to a different set of virulence factors (Navarro-Garcia, 2014). However, this was not the first reported LEE-negative STEC strain responsible for an outbreak. In 1994, an outbreak of diarrhea was reported in Montana caused by a LEE-negative STEC strain belonging to STEC serotype 104:H21. None of the human cases developed HUS. A similar outbreak was reported in 1999 in South Australia cause by a LEE-negative O113:H21 STEC strain, but in this case the infected persons developed HUS (Paton *et al.*, 1999). Furthermore, the STEC serogroup O91 belongs to the European “big five”, but possesses rarely *eae*. While this STEC serogroup appears less pathogenic compared the other serogroups of the “big five”, it has been associated with some sporadic cases of HUS (Maeda *et al.*, 2015). The limitations of the seropathotype scheme by Karmali were made clear, since *E. coli* O104:H4 had been classified as seropathotype D. Therefore, the EFSA Panel on Biological Hazards (BIOHAZ) concluded that this scheme does not define pathogenic STEC nor does it provide an exhaustive list of pathogenic serotypes (EFSA, 2013b). Still, the assessment of the clinical and public health risks associated with different STEC strains remains of utmost importance.



## 2. *Prevalence and transmission of Shiga toxin-producing Escherichia coli*

### 2.1. *Prevalence*

STEC is an important food-borne pathogen worldwide. In the northern hemisphere its significance has been shown in Canada, North America, Europe and Japan. In the latter two a clear seasonal variation was observed, with more reported cases during summer. But STEC is also of major concern in countries in the southern hemisphere, such as Argentina, Australia, Chile and South Africa (Nataro and Kaper, 1998). Yearly estimates indicate a global incidence of 1 176 854 acute cases, with 128 fatalities. The impact of STEC is often more severe in children, especially with HUS development and death occurring more frequently in children between the ages of 0 and 4 years (Majowicz *et al.*, 2014; WHO, 2015). Surveillance systems are in place to recognize and manage specific outbreaks, based on the laboratory confirmed cases. However, the methods used are dependent on routine diagnostic laboratories and can therefore still lead to underestimations of the incidence of STEC. In Europe, the surveillance system is coordinated by the European Centre for Disease Prevention and Control (ECDC). All gathered data concerning STEC infections is published annually in the “European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks” (Caprioli *et al.*, 2014). In the period 2008-2014 an increasing trend of reported cases can be observed, with a clear seasonal variation (Figure I-2).

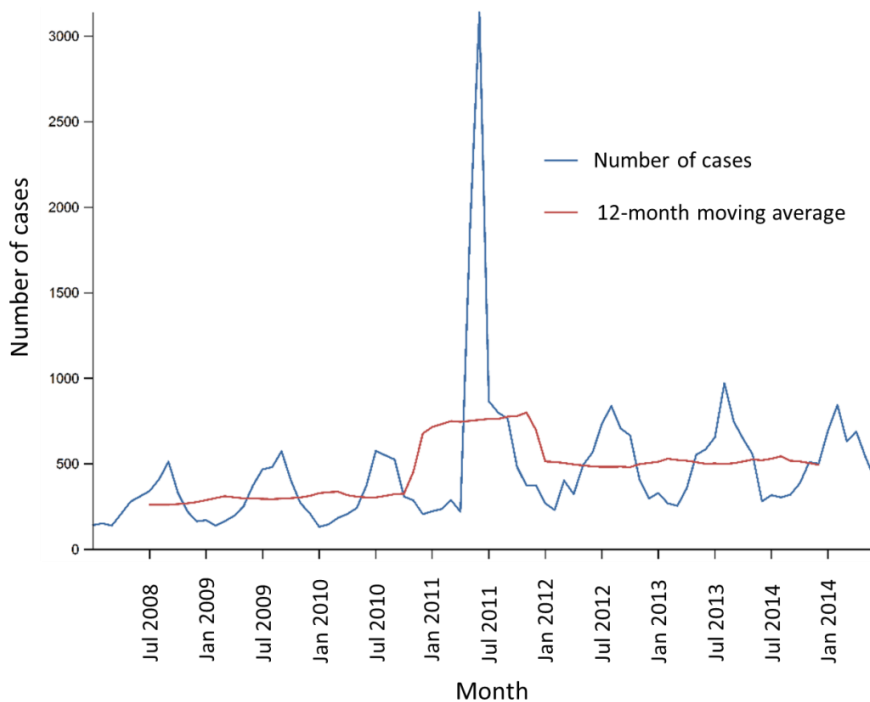


Figure I-2. Trend in the reported human STEC cases in Europe, by month of reporting over a period of 2008-2014 (EFSA, 2016).

The most prominent peak in the summer of 2011 was due to the O104:H4 STEC outbreak in Germany. After this outbreak a general increase in awareness and detection of non-O157 STEC strains can be observed, explaining in part the increasing trend of reported STEC cases. The most prevalent serogroup is still O157 followed by O26, O103, O145, O91 and O111 (EFSA, 2016, 2015, 2014).

In the United States, the surveillance system is coordinated by the Centre for Disease Prevention and Control (CDC). The CDC plays a more active role in the surveillance of STEC cases compared to the ECDC, and is routinely communicating with the diagnostic laboratories. In contrast with the ECDC approach, clinical diagnoses are always linked to the outbreaks (Table I-1). To facilitate the identification of transmission routes and outbreak sources, the STEC strains are routinely subtyped by performing a PulseNet standardized pulsed-field gel electrophoresis (Caprioli *et al.*, 2014) (see 5.2 Genotyping).

**Table I-1 Reported multi-state STEC outbreaks by CDC following the O104:H4 STEC outbreak in Germany during 2011 (CDC, 2016, 2015, 2014, 2013, 2012, 2011).**

Outbreak strain	Date <sup>1</sup>	Outbreak source	Complicated with HUS	Multi-state outbreak
O104:H4	May 2011	Fenugreek sprouts <sup>2</sup>	HUS	6 states
O157:H7	October 2011	Romaine lettuce	HUS	9 states
O145	April 2012	Unknown	No	5 states
O157:H7	October 2012	Organic spinach and spring mix	HUS	5 states
O26	December 2012	Clover sprouts	No	4 states
O121	December 2012	Frozen mini meals and snacks	HUS	9 states
O157:H7	October 2013	Ready-to-eat-salad	HUS	4 states
O121	May 2014	Clover sprouts	No	5 states
O157:H7	May 2014	Ground beef	No	4 states
O111	June 2014	Cabbage salad	HUS	7 states
O26	October 2015	Unknown	No	2 states
O157:H7	October 2015	Chicken Salad	HUS	7 states
O157:H7	April 2015	Dairy	HUS	1 state
O157:H7	January 2016	Alfalfa sprouts	No	2 states

<sup>1</sup> Date of the outbreak was determined at the onset of the first reported clinical illness, <sup>2</sup> outbreaks in US associated with travel to Germany

## 2.2. *Animal reservoir*

The epidemiology of STEC includes a reservoir in the intestinal tract of cattle, sheep, goats, pigs, cats, dogs, chickens and gulls. A broad variety of STEC strains can be found in these animals. These animals can be divided into three groups, first the *natural reservoir* which includes those animals susceptible to colonization and able to transmit the disease. The second group shares the same characteristics as the natural reservoir, but unable to maintain the colonization once the STEC source is absent and are therefore called the *spillover hosts*. And finally the *dead-end*

*group*, consisting of those organisms unable to transmit the pathogen other than by being consumed (Persad and Lejeune, 2014).

### 2.2.1 Ruminants

The most important natural reservoir associated with human illness is cattle. These animals are exposed to STEC through other animals, contaminated feed or water. While Stx is able to bind to the bovine epithelial cells of the intestinal tract and to a specific subset of kidney cells, the lack of the vascular Stx receptors, the globotriaosylceramide-3 receptor (Gb3), ensures that cattle are not affected by the infection (Hoey *et al.*, 2002). Nevertheless, some STEC strains can cause diarrhea in calves, most of these strains are LEE-positive EHEC strains and belong to the serogroups O5, O26, O111 and O118. All of these serogroups have been associated with human disease, however, the absence of O157 is remarkable (Mainil and Daube, 2005). The main colonization site of cattle is the terminal part of the intestinal tract, namely the recto-anal junction (RAJ). The shedding of STEC occurs sporadic and only during short periods of time. The amount can vary between a few colony-forming units (CFU) to  $10^9$  CFU per gram feces. Moreover, some animals may be more persistent carriers of the pathogen or shed at higher levels (at least  $10^4$  CFU per gram feces) for a longer period (>10 days) than others. These so-called “super-shedders” have a major impact on the on-farm prevalence and transmission, and food contamination (Duffy *et al.*, 2014; Matthews *et al.*, 2006). The prevalence of STEC in the bovine herd is difficult to measure, since seasonal variation, with a peak during the warmer months, and intermitted shedding can make the herd infection rates highly variable. *stx*-positive beef cattle on global scale varies from 0.2 to 27.8% for O157 STEC and 2.1 to 70.1% for non-O157 STEC (Hussein and Bollinger, 2005) and for dairy cattle from 0.2 to 48.8% for O157 STEC and from 0.4 to 74.0% for non-O157 STEC (Hussein and Sakuma, 2005). Other ruminants, such as sheep, goat, deer and buffalos, are also recognized as natural reservoirs and may be relevant depending on the region (Persad and Lejeune, 2014).

### 2.2.2 Other animals

*Swine* are also reported often to carry STEC belonging to various serotypes. In contrast to the ruminants, these animals possess the globotetraosylceramide (Gb4) receptor with special affinity for a particular Stx2 subtype Stx2e (see 3.2.3 Shiga Toxin). Both ETEC and Stx2e-STEC have been implicated to cause post-weaning diarrhea and edema disease. *stx2e*-positive STEC is most frequently found in porcine feces. Because of the lack of other virulence factors, these strains seldom cause human disease (Kaufmann *et al.*, 2006; Waddell *et al.*, 1998). A clear example of spillover hosts are *birds*. While the prevalence of STEC carrying birds is rather low, the potential

risk is significant. The bird-to-bird and bird-to-animal transmission, in addition to the ability of transferring STEC over great distances may warrant more attention (Kauffman and Lejeune, 2011). Similarly, *insects* can act as important vectors for STEC, where it remains viable for at least three days and can be transmitted to other animals and even to exposed food surfaces (Persad and Lejeune, 2014; Wasala *et al.*, 2013). Some reports have been made of STEC contaminated *fish and shellfish* caught downstream of intensive livestock activities. So far there is no proof that in these cases STEC can be transmitted to other organisms, therefore fish and shellfish are classified as dead-end hosts. These organisms may cause a public health risk when improperly handled or prepared (Balière *et al.*, 2015; Persad and Lejeune, 2014).

### 2.3. Food

The widespread STEC in animals can be transmitted to humans through many different routes, either by direct contact with STEC contaminated fecal material, or indirectly via consumption of fecally contaminated food, particularly from bovine origin (Figure I-3). These food products become contaminated mostly due to cross-contamination by dirty hides and leakage of intestinal content in slaughterhouses (Barco *et al.*, 2015). Besides undercooking and raw consumption, cross-contamination due to unhygienic handling of food in the kitchen was suggested as a leading cause of STEC transmission.

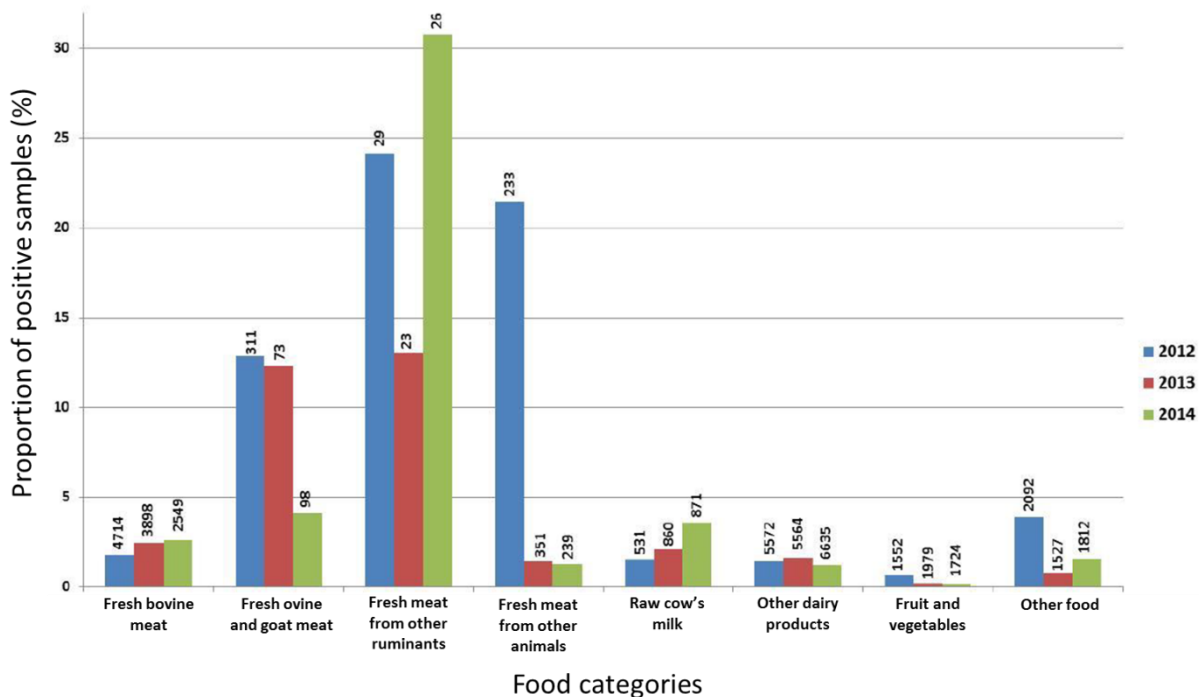


Figure I-3. The proportion of STEC positive samples reported in the EU, per food category, excluding all selective samplings, outbreak and clinical investigations (EFSA, 2016).

The transmission is however not limited to cattle and bovine meat, other food has been linked to the STEC outbreaks. Products with a low pH, such as apple juice, dairy products and fermented salami, allow the survival of STEC. Pasteurization is usually sufficient to eliminate the pathogen, however, recontamination or inadequate pasteurization are often the cause of food-borne outbreaks (Duffy *et al.*, 2006). Since modern dietary advice has begun promoting the consumption of fresh produce such as fruit and vegetables often consumed raw, an increasing number of outbreaks associated with the consumption of these products is reported (Hou *et al.*, 2013; Sivapalasingam *et al.*, 2004). There are many potential sources of contamination for fresh produce. Pre-harvest contamination occurs during growth due to contaminated soil, fertilizers and irrigation water, or occurs during harvest, and is probably the main concern in terms of food-borne pathogens. Therefore, a relatively high total bacterial count can be expected pre-harvest. *E. coli* O157:H7 has been shown to survive two years on seeds, such as butterhead lettuce seeds, while maintaining their ability to resuscitate and proliferate in the sprouted seeds (Van der Linden *et al.*, 2013). Especially leafy greens, such as spinach and lettuce, are susceptible to contamination, since they grow in close contact to the ground. In addition, they are often consumed raw after little cleaning (Kase *et al.*, 2012). Especially in developing countries crops are often irrigated with untreated waste water. However, post-harvest contaminations may also occur during washing, slicing, soaking, packaging and preparation (Castro-Rosas *et al.*, 2012). The “ready-to-eat” bagged vegetables that claim to be thoroughly washed have been implicated in foodborne outbreaks (Kase *et al.*, 2012). Even after processing and preparation of these fresh products, total bacterial counts were reported ranging from  $10^3$  to  $10^7$  CFU per gram, with high deviation between lots or even within the same lots (Feng, 2014; Soriano *et al.*, 2000). Since the low infective dose of STEC, the detection of small amounts of STEC is crucial. The detection in fresh produce is even more challenging due to the heterogeneous distribution of the pathogen over the surface, the broad spectrum of fresh produce and the high amount of microbiota competing and overwhelming the target pathogen (see 4.4 Cultural methods) (Feng, 2014).

Water is also an important source of STEC, with recreational water, drinking water, surface and ground water and indirectly irrigation water already been associated with outbreaks (Nataro and Kaper, 1998; Saxena *et al.*, 2015). The survival mechanism of STEC in water is probably based on their ability to tolerate a range of biological, physical and chemical stress, such as temperature, solar radiation, predation and nutrient availability. *E. coli* O157:H7 was reported to survive for two months in samples collected from four types of water, lakes, river, fecal contaminated puddle and animal drinking water (Avery *et al.*, 2008).

### **3.      *Colonization, Stx-converting bacteriophages and virulence genes of STEC***

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#### **3.1.      *Colonization***

During the colonization process STEC will be able to overcome the host defense and establish itself in the intestine. The first barrier is the low pH environment of the stomach. However, STEC, as all *E. coli*, has the innate ability to resist acid conditions. Once in the intestine, the thick mucus layer at the surface of intestinal epithelial cells (enterocytes) must be penetrated. Flagellae are used to propel the pathogen within the mucus layer and enzymes to alter the viscosity of the layer (McGuckin *et al.*, 2011). The first contact with the enterocytes is with the protruding microvilli. It was suggested that bacterial fimbrial pili can extend and connect with the microvilli. With the retraction of these pili the pathogen is brought into close proximity with the enterocyte (Humphries *et al.*, 2010). Others suggest that the hollow filamentous structure, which is part of the type III secretion system (T3SS) plays a role in the initial adhesion to the enterocyte (Sekiya *et al.*, 2001). Still other studies suggested the adhesive properties of the flagella to be involved (Giron *et al.*, 2002; Mahajan *et al.*, 2009). Only after all these barriers are overcome, the intimate adherence mechanisms can be initiated. The adherence mechanisms are distinctly different between LEE-positive and LEE-negative STEC strains.

##### **3.1.1      LEE-positive STEC strains**

The LEE encodes for a type III secretion system (T3SS), an outer membrane protein intimin and the translocated intimin receptor (tir). The T3SS is a key virulence factor in many Gram-negative intestinal pathogens. A syringe-like structure is formed on the inside of the bacterial cell and penetrates both inner and outer cell membranes and invades the membrane of the host cell. The T3SS forms a pathway for the export of effector proteins, encoded by the LEE and others, to the cytoplasm of the host cell. One of the first effector proteins to pass is Tir. Tir will be inserted into the membrane of the host and expose its intimin-binding site extracellularly. Once the intimin on the bacterial surface binds to this receptor a firm bacterial-host cell attachment is established. The transferred proteins will initiate a number of signaling cascades that will induce the host cell to rearrange its cellular architecture. This causes the collapse of the microvilli and the accumulation of cytoskeletal proteins into columnar protrusions beneath the attached bacteria, leading to the formation of the typical pedestal (Figure I-1 B) (Gyles, 2007; Law and Guttman, 2014) .

### 3.1.2 Other colonizing factors

STEC possesses a large number of other proteins responsible for the adhesion of the pathogen to the host. As already mentioned, a number of LEE-negative STEC strains have been described to colonize and cause HUS. Therefore, the presence of LEE-genes is not essential to ensure colonization (Paton *et al.*, 1999). Recently, a few adhesins have been identified aiding the adherence mechanisms of both LEE-negative and LEE-positive STEC strains. Several gene clusters have been identified that encode for both fimbrial adhesins and nonfimbrial adhesins. However, in many cases only a few of these genes were expressed (Gyles, 2007). The fimbrial adhesins consists of proteins, such as the long polar fimbriae (Lpf) and curli. Lpf was first identified in *Salmonella enteritica* serovar Typhimurium, but can be found in a wide range of pathogenic *E. coli*. While, Lpf allows mainly adhesion to intestinal cells, the thin fibers that form Curli also allow colonization of other surfaces in the environment (McWilliams and Torres, 2014). The nonfimbrial adhesins include a group of autotransporter adhesins. These proteins are able to span the bacterial inner and outer membrane and be exposed on the surface, independent of any other bacterial protein secretion system (Leyton *et al.*, 2011). Well-known autotransporter proteins are the serine protease P (EspP) and STEC autoagglutinating adhesin (Saa). EspP is a member of the autotransporters of the Enterobacteriaceae. Besides the adhesive abilities, the enzymatic activity of this protein may help in the immunomodulation of the host response (Farfan and Torres, 2012). Saa is the first adhesin identified in a LEE-negative STEC strain by Paton *et al.* (2001). This protein showed a low degree of similarity to the adhesin YadA of *Yersinia enterocolitica*. Other nonfimbrial adhesins are flagella, IrgA homologue adhesin (Iha) and EHEC factor for adherence (Efa1). As mentioned above flagella can interact with the protective mucus layer. In addition they are, at least in part, responsible for the adhesion to the surface of leafy greens (McGuckin *et al.*, 2011). Iha is an outer membrane protein encoded by an adherence-conferring gene similar to the iron-regulated gene A (*irgA*) of *Vibrio cholerae* (Tarr *et al.*, 2000). However, this gene seemed to be restricted to *E. coli* O157:H7 and its actual role in the adhesion process needs to be verified (McWilliams and Torres, 2014). Efa1 is an adhesion factor that is reported to aim in the colonization of the bovine intestine (Gyles, 2007).

The most notorious LEE-negative STEC strain, namely the outbreak strain O104:H4 STEC belonging to the new subgroup of Enterohemorrhagic *E. coli* (EAHEC), carried the pAA plasmid. This plasmid encodes for the aggregative adherence fimbriae (AAF) required during the intestinal colonization and as in all EAHEC, the colonization is regulated by the *aggR* gene (Navarro-Garcia, 2014).

### 3.1.3 Plasmid pO157

By definition all EHEC strains possess a large plasmid pO157, with some similarities to the EAF plasmid of EPEC. pO157 encoded several virulence factors, such as a hemolysin typical for EHEC, namely (EHEC) enterohemolysin (Paton and Paton, 1998). This enterohemolysin is related to the  $\alpha$ -hemolysin produced by many pathogenic and commensal *E. coli*. However, while  $\alpha$ -hemolysin is chromosomally encoded by the *hlyC*, *hlyA*, *hlyB*, and *hlyD* genes, the enterohemolysin is encoded by an operon located on the large plasmid containing *ehxC* (EHEC-*hlyC*), *ehxA* (EHEC-*hlyA*), *ehxB* (EHEC-*hlyB*) and *ehxD* (EHEC-*hlyD*) genes (Schmidt *et al.*, 1995). This enterohemolysin works as a pore-forming cytolysin on eukaryotic cells and releases hemoglobin from red blood cells. Furthermore, it can cause damage to human microvascular endothelial cells, suggesting a possible role in the pathogenesis of bloody diarrhea and HUS (Aldick *et al.*, 2007; Uhlin *et al.*, 2014). The plasmid also harbors *katP*, *espD*, *espP* and *toxB*. The latter is a homologue to *Clostridium difficile* toxins A and B and contributes to the adhesion of a wide variety of STEC strains (Gyles, 2007).

The LEE-negative STEC strains associated with human illness also seemed to harbor a large plasmid which encodes EHEC hemolysin (*ehx*). This plasmid is of a similar size to the EHEC plasmid, both plasmids are evolutionarily distinct. This plasmid encodes the autotransporter proteins Saa and Sab (Paton *et al.*, 2001).

## 3.2. *Shiga toxin production*

Once STEC is able to adhere and colonize the large intestine, the varying degree of intestinal tissue damage leads to mild to severe diarrhea and dehydration. However, unlike *Shigella dysenteriae* and EIEC, STEC will not invade the enterocytes. Instead it will secrete Shiga toxins in the intestinal lumen (Nataro and Kaper, 1998).

### 3.2.1 Stx-converting bacteriophage

Smith and Lingwood (1971) demonstrated the possibilities of transferring virulence factors, such as toxin production, from a diarrheagenic *E. coli* to a non-pathogenic *E. coli*, suggesting the presence of mobile genetic elements. In accordance the Stx1 and Stx2 production of STEC seemed associated with two distinct highly mobile genetic elements (Strockbine *et al.*, 1986). Both are encoded on prophages integrated into the *E. coli* chromosome, which ensure horizontal transfer. On the other hand, Stx of *Shigella dysenteriae* are chromosomally encoded and are not transmissible (Johannes and Römer, 2010). However, McDonough (1999) has shown that originally the Stx of *Shigella dysenteriae* was also encoded by a prophage, but due to deletions during sequence rearrangement, the gene sequences became chromosomally anchored. Probably the



same may have happened for the Stx2e encoding gene, which is also located chromosomally (Juillot and Römer, 2014). Each Stx-converting double-stranded DNA bacteriophage or in short Stx-phage encodes either for Stx1 or Stx2, and each Stx type has several subtypes. All Stx-phages have a similar genetic organization and regulation, and share a common gene pool. The position of the *stx* genes is conserved in the different prophages and are located in the late gene region, downstream of the late promoters and upstream of the lysis cassette (Tyler and Friedman, 2004). The Stx-phages largely resemble lambdoid phages ( $\lambda$ -phages). Furthermore, the Stx-phages represent a very heterogeneous group as, despite the similar genetic organization, most genes will vary in sequence (Tyler and Friedman, 2004). In addition, an exchange of genes between lambdoid phages occurs frequently. Therefore, a Stx-phage may carry additional virulence factors. The Stx-phages have been described as belonging to several different viral families, such as *Siphoviridae*, *Podoviridae* and *Myoviridae*, each displaying their typical morphology (Ackermann, 2001). As typical for all  $\lambda$ -phages, specific integration sites in the bacterial chromosome are used for the integration of the Stx-phages. Certain integration sites have been described (*wbrA*, *yehV*, *sbcB*, *yecE*, *argW*, *ssrA* and *prfC*), thus the presence of a specific site in the host is important. Furthermore, dependent on the type of Stx-phage a specific integration site will be preferred (Ogura *et al.*, 2007). However, when the preferred site is already occupied by a Stx-phage or truncated, a secondary integration site can be used. So Stx-phages are able to superinfect a bacterial cell with two or more similar or different Stx-phages.

The main characteristic of all  $\lambda$ -phages is the choice between a lysogenic and lytic pathway. During lysogenic pathway the phage integrates its genome into the bacterial host genome as a prophage. In a lysogenic bacterium no Stx is produced because the flanking gene promoters are repressed. The lytic pathway results in a Stx-phage induction, during which the bacteria are induced to intracellularly assemble new phage particles. Since the *stx* gene promoters are no longer repressed, the Stx-phage induction is linked to the production of Stx. These new Stx-phages and toxins are released with the lysis of the host cell (Neely and Friedman, 1998; Smith *et al.*, 2012).

Several triggers have been described to activate the lytic cycle of the Stx-phages and potentially affecting the outcome of the STEC infection. While still little is known about the exact mechanism of spontaneous induction of Stx-phages, the Stx-phages and in particular *stx2*-phages are more prone to spontaneous induction compared to other  $\lambda$ -phages. In general, an events that damages bacterial DNA or inhibits bacterial DNA synthesis activates the bacterial SOS response which leads to the induction of Stx-phages. Antibiotics are an important group of triggers, either by damaging bacterial DNA (e.g. mitomycin C) or inhibiting bacterial DNA

synthesis (e.g. quinolones). But also UV light, irradiation and high hydrostatic pressure can activate the SOS response (Muniesa and Schmidt, 2014). Therefore, the lysogenic state is preferable for the host and the Stx-phage. Moreover, some Stx-phages protect their lysogenic host bacteria from infection by other bacteriophages or might even protect the bacteria from severe stress (Tyler and Friedman, 2004). Often two Stx-phages are integrated in one bacterial cell (double lysogenic), however, these prophages are less likely to be induced. Serra-Moreno (2008) suggested that the reduction of phage induction in double lysogenic bacteria was due to the conserved repressor proteins of both prophages working together to avoid induction. On the other hand, Fogg *et al.* (2012) demonstrated an increased Stx expression in the double lysogenic bacteria compared to single lysogenic bacteria. However, the amount of Stx production is limited because the protein resources within the bacteria are insufficient.

### 3.2.2 Free-living Stx-phage

While, the cyclic pathway of the prophages leads to the expression of the toxin and therefore greatly influences the course of the illness, the release of new Stx-phages is also of importance. These Stx-phages spread in the environment, such as rivers and sewage systems, where they can persist for a long time. Moreover, they are able to survive certain intervention measures that will eradicate their bacterial hosts (Dumke *et al.*, 2006). Still little is known about the prevalence of the Stx-phages in the environment, because the routine enumeration practices are insufficient for accurate measurements of Stx-phages. Unlike most phages, the Stx-phage often produces poorly visible plaques during plaque assays. Furthermore, the host bacteria might remain in the lysogenic state. Culture-independent methods based on qPCR can overcome these drawbacks, but will be unable to distinguish infective from defective non-infective phage (Rooks *et al.*, 2010).

Martínez-Castillo *et al.* (2013) demonstrated that *stx2*-phages were present in feces of 62% healthy human patients. It can be assumed that part of the phages was ingested in their free form present in water and food. However, their presence in water environments was not always associated with the occurrence of fecal contamination. This suggests the presence of other Stx-phage sources in the environment (Martínez-Castillo and Muniesa, 2014). Furthermore, free-living Stx-phages have been detected in food, such as beef and salad, even though these products were conform to the present process hygiene microbiological criteria under EU regulation. This may suggest a limitation of the present legislation concerning the presence of phages in food (Imamovic and Muniesa, 2011).

The main question about Stx-phages remains unanswered: what is the benefit for *E. coli* for harboring these phages? By carrying these Stx-phages the *E. coli* cells are under a constant risk of being eliminated when the lytic pathway is activated. Therefore, a non-inducible defective

prophage, such as *Shigella dysenteriae* harbors, might be more beneficial to the bacteria. On the other hand, it would be less favorable for the Stx-phages, as they will be unable to infect other *E. coli* and will no longer be able to exchange genetic material. Therefore, an equilibrium was found among the potential risk and possible advantages for the bacteria (Muniesa and Schmidt, 2014). Some advantages for the bacteria have been described, such as promoting the colonization in cattle. A protective function of prophages against bacterivorous protozoa has also been suggested, especially against protozoa in the rumen. Moreover, these protozoa are killed by the Stx toxins. However, in order to release these toxins the lytic pathway must be activated, resulting in the death of the host cells. Thus the individual bacteria will not survive, while the overall population of lysogenic bacteria will benefit from the induction of a part of their population. The animals colonized by STEC lacking the vascular Stx specific (Gb3/Gb4) receptors, will be not susceptible for the toxin (Lainhart *et al.*, 2009). These findings suggest that the integration of a Stx-phage in *E. coli* facilitates STEC survival in animals, such as ruminants and that the human infection with STEC and its outcome might be rather accidental (Muniesa and Schmidt, 2014).

### 3.2.3 Shiga Toxin

STEC can produce either Stx1 or Stx2, or both when multiple lambdoid prophages are integrated in the chromosome. Stx1 can be neutralized using antibodies active against Stx produced by *Shigella dysenteriae* type 1, because both toxins only differ in one single amino acid. Stx2 cannot be neutralized by these antibodies (O'Brien *et al.*, 1983; Strockbine *et al.*, 1988). Moreover, within one Stx toxin type differences in biological activity, such as serological activity, receptor binding and interaction with intestinal mucus, were observed. Thus, each toxin type seemed to contain several subtypes, denoted with a small Arabic letter following the main type name. Based on a subtyping PCR method three and seven gene subtypes were identified for *stx1* (*stx1a*, *stx1c*, *stx1d*) and *stx2* (*stx2a* to *stx2g*), respectively (Harada *et al.*, 2015). Since certain Stx subtypes are more frequently associated with human illness, a consistent nomenclature for the *stx* subtypes is clinically relevant (Scheutz *et al.*, 2012). Both Stx1 and Stx2, are members of the AB<sub>5</sub> toxin family, like also cholera toxin and LT toxin associated with ETEC. These toxins consist of two subunits, the A-subunit, which is a monomer and is responsible for the enzymatic activity and the B-subunit, which consist of five identical monomers and binds specific receptors (Figure I-4 A). The globotriosylceramide (Gb3) receptor functions as the specific receptor for both Stx1 and Stx2, only Stx2e binds specific to the globotetraosylceramide (Gb4) receptor. The toxin is internalized in the host cell via endocytosis, followed by retrograde transport of the toxins through the Golgi apparatus to the endoplasmic reticulum (ER) (Johannes and Römer, 2010). During transport the

A-subunit is proteolytically cleaved, which results in a A1-fragment with increased enzymatic activity and a small A2-fragment (Garred *et al.*, 1995). The A1-fragment inhibits the protein synthesis by cleaving the 28S rRNA of the eukaryotic ribosomes. The inhibition of protein synthesis will lead to an accumulation of unfolded or badly folded proteins in the ER, resulting in an ER stress response. This response induces apoptosis or “programmed cell death” in these cells (Figure I-4 B) (Johannes and Römer, 2010; Lee *et al.*, 2005). Therefore, the expression of the Gb3 is a major determinant of the susceptibility to tissue damage. The action of Stx on endothelial cells of the small blood vessel in the colon and renal glomeruli are associated with the development of bloody diarrhea and HUS, respectively (Gyles, 2007).

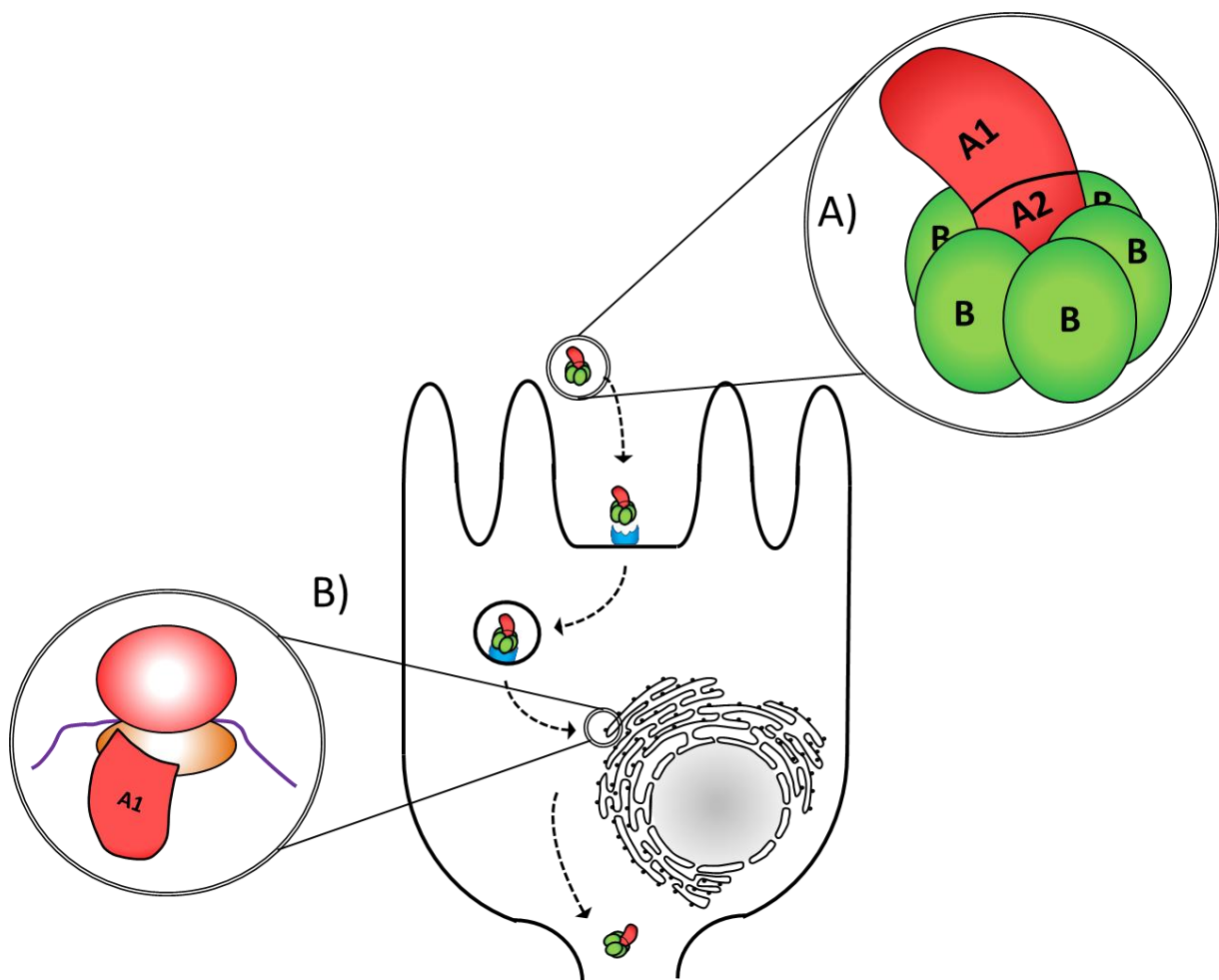


Figure I-4 A) Representation of the structure of Shiga toxin, consisting of one A subunit which is cleaved into fragments A1 and A2, and five B subunits. B) The internalization of the Stx toxin in the host cell via endocytosis, followed by transport to the endoplasmic reticulum (ER). The enzymatic active A1-fragment inhibits the protein synthesis of the eukaryotic ribosomes. Stx is released after apoptosis of host cell. Figure redrafted and adjusted from Johannes and Römer, 2010; Schüller, 2011

#### **4.      *Detection and isolation of STEC***

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As described above both the *E. coli* strains and the Stx-phages are members of very heterogeneous group. Furthermore, STEC can be present in a broad variety of animals, food and environmental niches. Therefore, the detection of STEC is challenging. In the last decades several methods have been described for the detection of all STEC. Two groups of detection methods are based on the identification of the Shiga toxins via cytotoxicity assays or immunological assays. A third group is based on the detection of the Shiga toxin-encoding genes via DNA-based methods. However, these methods can only indicate presumptive positive samples, the final culture-based methods are required to confirm the results.

##### **4.1.      *Tissue culture cytotoxicity assay***

This method is based on the original experiment proving the existence of toxin-producing *E. coli* with a cytotoxic effect on mammalian cells, namely Vero cells (Konowalchuk *et al.*, 1977). Since, this test not only detects Stx, but also verifies its cytotoxic effect, the tissue culture cytotoxicity assays remain the golden standard for Stx detection. During this method a supernatant preparation of the sample is brought into contact with a monolayer of mammalian cells, such as Vero or HeLa cells. After incubation the cytopathogenic effect is examined (Bettelheim and Beutin, 2003; Beutin and Fach, 2014). However, not all Stx show the same activity on the different cell lines (e.g. some Stx subtypes possess less affinity for HeLa cells) (Mainil and Daube, 2005). To improve the specificity of the test, Stx1 and Stx2- specific neutralizing antisera might be used, in order to verify that Stx is responsible for the cytotoxic effect and not another toxin present in the sample. For routine diagnostic testing, this method is expensive, difficult to standardize, labour-intensive and time-consuming (about one week). In addition, specialized facilities and trained personnel are required for this tissue cultures. Moreover, its use in the analysis of more complex matrices is rather limited due to the potential presence of active components and inhibitors (He *et al.*, 2011; Watarai *et al.*, 2001). Still, it remains a valuable method for any reference laboratory for detecting new Stx variants, which may be missed with specific immunological or DNA-based methods (Bettelheim and Beutin, 2003; De Boer and Heuvelink, 2000).

##### **4.2.      *Immunological assays***

A variety of commercial and non-commercial immunological assays have been developed since the importance of STEC was first recognized. A number of these assays are available as “ready to use” kits for routine diagnostic laboratories. In contrast to the culture cytotoxicity assay, these tests do not discriminate between biological active and inactive toxins. Furthermore, some

immunological assays fail to detect those strains that release low amount of toxins or none at all (Beutin *et al.*, 2007). The specificity of the anti-Stx antibodies used during the assay are of crucial importance in order to identify the various Stx subtypes (Burgos and Beutin, 2012). Since both Stx1 and Stx2 are immunologically distinct, no cross-reactivity of the anti-Stx1 and anti-Stx2 antibodies could be observed during these assays. Still, not all Stx subtypes are detected with the same efficiency (Karmali *et al.*, 1999). Samples are sometimes prepared by supplementing mitomycin C. This chemotherapeutic agent activates the lytic pathway of the prophage and therefore enhances the production of Stx (Hull *et al.*, 1993). Cultural enrichment of the sample can also stimulate the Stx production, especially in an enrichment medium, such as Brain Heart Infusion Broth (BHI). In contrast to most other enrichment broths, it contains a protein source of animal origin (Hussein *et al.*, 2008). Still, these assays are generally not sensitive enough to detect low amounts of Stx (He *et al.*, 2011). Besides the detection of Stx, a number of these tests are developed to identify the presence of specific O- and H-type antigens, to ensure a rapid detection of the most important STEC serotypes (De Boer and Heuvelink, 2000). Detection limit is approximately  $10^4$  to  $10^5$  CFU/ml, this is too low to recommend direct testing either on food or stool samples. An enrichment significantly increases the sensitivity, making it an appropriate assay for the detection of STEC in both food or stool samples (De Rauw *et al.*, 2016; Wang *et al.*, 2013). However, neither tissue culture cytotoxicity nor immunological assays yield isolates, which are essential to confirm the results. Therefore, before the STEC detection can be considered positive a cultural isolation is still needed (Cronquist *et al.*, 2012).

*Enzyme-linked immunosorbent assay (ELISA)* is a rapid and easy to use method and represents the most common form of immunological assay. For this method a supernatant preparation of the sample is transferred to a microtitre plate coated with Stx1 or Stx2 specific antibodies (Parma *et al.*, 2012). After incubation, to allow for the binding between Stx-antigen and antibody, the microtitre plate is washed and enzyme-labeled antibodies are added. The Stx-antigen is now captured between two sets of antibodies, when specific enzyme substrate is included, a discoloration can be observed. By assessing the color intensity with a spectrophotometer an indication concerning the amount of Stx present in the sample can be obtained. This method can only detect the presence of the toxin, whether STEC is present in the examined sample or not remains unknown (Bettelheim and Beutin, 2003).

*The immunochromatographic lateral flow test* shares a similar format with ELISA, but is faster and more user-friendly. The strips or sticks absorbent end is brought into contact with a liquid sample. This sample is directed by capillary action through a porous membrane that contains the specific anti-Stx antibody. These antibodies are labeled with a color particle (gold or latex). The antigen-antibody complexes are captured by another antibody at the test line, allowing the

color particles to concentrate and form a visible line. However, complex samples with different origins and chemical properties may need additional treatment, such as centrifugation, serial dilution and pH adjustment, to ensure proper analysis (Aldus *et al.*, 2003; Burgos and Beutin, 2012; Ngom *et al.*, 2010).

*Reverse passive latex agglutination assay* is mostly used to confirm presumptive STEC colonies from agar plates or culture filtrate. These plates are often supplemented with an agent to enhance Stx production. This fast identification method is based on latex beads coated with specific anti-Stx antibodies. When the sample is brought into contact with the coated beads the Stx will bind the antibodies leading to visual agglutination or flocculation in a few seconds (Karmali *et al.*, 1999; Medina *et al.*, 2012).

*Colony immunoblotting assay* might provide a single step solution for the confirmation of a larger number of presumptive STEC colonies. Blots are made from the isolation plates onto membranes. The colonies are detected by the ELISA procedure, using specific antibodies and identified by the immunopositive sites on the blot. *Colony hybridization assay* is based on the same principles as colony immunoblotting assay, but instead of using specific antibodies, a DNA-based probe is applied (Bettelheim and Beutin, 2003; De Boer and Heuvelink, 2000; Hull *et al.*, 1993).

### **4.3. DNA-based methods**

In the past decades PCR has become a very important tool for the detection of pathogens. While no longer specific antibodies or cell cultures are required, specific primers and probes become necessary in order to amplify specific sections of the target genes. These methods have become indispensable and are certainly more reliable compared to the immunological assays. However, the presence of free-living Stx-phages in a sample can also cause false positive results (Bettelheim and Beutin, 2003). Moreover, the presence of genes originating from dead cells or the potential loss of *stx* genes upon subculturing make data interpretations even more complex (Deisingh and Thompson, 2004).

#### **4.3.1 PCR assays**

*The conventional or first generation polymerase-chain reaction (PCR)* was a significant improvement compared to the previous methods, because the DNA sequence of interest could be amplified in about 1-2 hours. However, the many toxin subtypes each encoded by different *stx* genes hinders the use of an universal primer pair. Minimum one primer pair for *stx1* subtypes and two primer pairs for the *stx2* subtype were required to cover all known subtypes (Deisingh and Thompson, 2004). While the original PCR assays allowed the amplification of a single target,

multiplex PCR (mPCR) assays were developed capable of co-amplifying one or more targets. This assay made the combination of one or more primer pairs in one reaction possible. Besides the detection of *stx1* and *stx2* genes, these assays were able to include other virulence genes, such as *eae* and *ehx*, or serotype-specific genes (*wzx*, *wzy*, *fliC*) (Beutin and Fach, 2014; Deisingh and Thompson, 2004; Wang *et al.*, 2002). For accurate interpretation of the PCR results separation of the amplified DNA molecules is required, using gel electrophoresis. In order to overcome the time-consuming limitations, such as gel electrophoresis, variations on the PCR principle were developed. Loop-mediated isothermal amplification (LAMP) is such a promising amplification assay, which uses 4 to 6 primers to amplify 6 to 8 distinct regions. The amplification occurs at a constant temperature (60-65°C) within one hour and is read by visual inspection or turbidity of the product (Dong, Cho, 2014).

Gel electrophoresis can also be avoided when an additional fluorogenic dye or probe is included to the PCR mixture. By measuring the fluorescence after every successive PCR cycle, the amplification process of the target gene can be monitored in the time. Therefore, this *second generation PCR* is called *real-time PCR*. This approach has become the universal method used for STEC detection in clinical cases, food and environmental samples (Beutin and Fach, 2014). A large number of commercial and non-commercial real-time PCR-based assays targeting *stx1* and *stx2* have been described in the past decades. However, these assays showed variable sensitivity and specificity, especially for some *stx2* subtypes. Furthermore, the fluorogenic dye SYBR Green proved less accurate compared to a specific TaqMan-based probe (Chui *et al.*, 2010). Both the International Organization for Standardization (ISO) and the U.S. department of Agriculture (MLG 5B.05) have developed a reference method for the detection of the most important STEC serogroups from food based on real-time PCR (Figure I-5). Both real-time PCR assays use one primer pair for the detection of the *eae* gene and one primer pair for both *stx1* and *stx2* (excluding *stx2f*). Therefore, neither can differentiate between the two *stx* types. When a sample tests positive for *stx*, the presence of certain STEC serogroups is investigated. The ISO/TS 13136:2012 includes the “big five” STEC serogroups (O157, O26, O103, O111, O145), while the U.S. also includes STEC serogroup O45 and O121, the “big seven” (ISO, 2012; U.S. department of Agriculture, Laboratory QA/QC, 2014).

While the PCR assays are very reliable for the analysis of cultures of bacterial colonies, the assay is subject to interfering background and inhibitory factors from matrix samples. Therefore, the matrix type of the sample and sample preparation may have major influence on the final outcome of the PCR (Nataro and Kaper, 1998). These interfering factors are common for the detection of STEC in food matrices, such as on leafy greens. Since, rinsing these vegetables might be unreliable to remove the attached STEC organisms, a blend procedure is recommended. While



the sample becomes homogenized, the DNA might degrade and PCR inhibitors, such as plant polysaccharides and phenolic compounds, may be released (Kase *et al.*, 2012). Recently, a third-generation PCR or *digital PCR* (dPCR) has been developed. The technique is based on partitioning of the PCR sample into many thousands of droplets or chambers. The PCR amplification occurs in each partition. The fluorescence signal of each partition is individually counted. Since the dPCR is, like the first generation PCRs, an end-point PCR it is suggested to be more flexible concerning sample quality and thus less prone to PCR inhibition (Hindson *et al.*, 2013; Pinheiro *et al.*, 2012).

A remaining bottleneck concerning PCR is the very small fraction of the original sample that can be tested. Unless an additional concentration step is used, the sensitivity of the method is hereby drastically reduced (He *et al.*, 2011).

#### 4.3.2 Combination assay: immuno-PCR

Some STEC strains are associated with a low toxin release. Since, the immunological methods described above are insufficiently sensitive to detect these amounts of toxin, a combined method was suggested. The immuno-PCR method combines the antibody capture with DNA amplification (Zhang *et al.*, 2008). The main principles of ELISA are employed, an antigen-antibody complex is formed, but instead of using an enzyme-conjugated antibody, the antibody is labeled with a DNA fragment. During PCR this DNA fragment is amplified (Mehta *et al.*, 2014). While the sensitivity was already significantly improved using the combination of ELISA and the conventional PCR (Fach *et al.*, 2001), the immuno-real-time-PCR emerged as the most sensitive assay, with reported detection levels of 0.1 pg/ml of purified Stx2 (He *et al.*, 2011; Zhang *et al.*, 2008).

### 4.4. *Cultural methods*

The recovery of an isolate from suspected contaminated food is still a fundamental requirement before any public health actions can be initiated. Following cultural enrichment, a small aliquot of the food sample is plated on a suitable isolation agar. Cultural isolation remains the only method to confirm the results of the rapid screening tools described above. By verifying the presence of the *stx* genes in the obtained isolates a sample can be declared positive. Moreover, the isolated STEC strains allows further characterization (Kase *et al.*, 2015).

#### 4.4.1 Cultural enrichment

Since the infective dose of STEC is reported to be low, low numbers of STEC cells in matrices such as food, may have a large impact on public health. Therefore, an initial cultural enrichment

step is essential to allow the growth of STEC in the matrix to a detectable level. Moreover, these few cells are often found together in the presence of high numbers of competitor organisms, especially closely related species of *Enterobacteriaceae* might interfere during enrichment (De Boer and Heuvelink, 2000; Smith and Fratamico, 2012). Hence, the enrichment step should ensure the STEC cells' growth while inhibiting the competitor organisms. A suitable medium has been described for the selective enrichment of *E. coli* O157:H7, namely 24 hours at 41.5°C in tryptone soy broth (TSB) modified with bile salt no. 3 and novobiocin (20 mgL<sup>-1</sup>) (Figure I-5) (ISO, 2001). However, no such medium is formulated for non-O157 STEC strains. A multitude of media, supplements and incubation conditions have been evaluated for the growth of non-O157 STEC (Table I-2). However, as the different STEC strains react differently when exposed to these selective components, the development of one reference medium remains challenging (Baylis, 2008).

**Table I-2. Most commonly used types of enrichment media for the detection and/or isolation of STEC in food (Baylis, 2008).**

Enrichment medium	abbreviation
Tryptone soy broth	TSB
<i>E. coli</i> broth	EC
Buffered peptone water	BPW
Brain heart infusion broth	BHIB
Brilliant green bile broth	BGGB
MacConkey broth	MCB
Gram-negative broth	GN
Minerals modified glutamate broth	MMGB
Lauryl sulfate tryptose broth	LSTB
Universal preenrichment broth	UPB

Vimont *et al.* (2006) compared 380 experiments described in the literature and noted that TSB, EC and BPW were used most frequently. In almost half of the experiments the enrichment media were supplemented with selective components to enhance STEC detection and isolation. Bile salts and novobiocin were the most commonly used selective agent and antibiotic, respectively. *TSB* is a highly nutritious medium used for the detection and/or isolation of many bacterial species, including foodborne pathogens. It is probably due to their popular use in enrichment protocols of other foodborne pathogens that it was also adopted for STEC detection. Doyle et Schoeni (1987) reported the increased performance of TSB after supplementation with novobiocin (20 mgL<sup>-1</sup>) (TSB+n) for the isolation of *E. coli* O157 in meat samples. This medium became a widely accepted method of enrichment of *E. coli* O157. However, a significant number of non-O157 STEC strains appeared to be susceptible to this level of novobiocin (Vimont *et al.*, 2007). In order to further improve the detection and isolation of *E. coli* O157, TSB+n was modified

by eliminating the novobiocin and including the antibiotic combination of vancomycin (8 mgL<sup>-1</sup>), cefsulodin (10 mgL<sup>-1</sup>), and cefixime (0,05 mgL<sup>-1</sup>) to inhibit Gram-positive bacteria, *Aeromonans*, and *Proteus* spp., respectively and was called EHEC Enrichment Broth (EEB) (Weagant *et al.*, 1995). *EC* is an enrichment medium often used for the detection of *E. coli* in general, because it provides as carbohydrate source lactose. Lactose can be fermented by *E. coli* in contrast with many members of the Enterobacteriaceae (Hussein and Bollinger, 2008).

*BPW* was originally proposed for the detection of stressed *E. coli* O157, with or without the supplementation of antibiotics (Hussein and Bollinger, 2008). However, these stressed strains may react differently in certain media with supplements or more selective incubation conditions (Smith and Fratamico, 2012). Therefore, a pre-enrichment step in the absence of selective agents to resuscitate the stressed bacteria may be preferable prior to selective enrichment (Hara-Kudo *et al.*, 2000a). Some supplementations have been proposed to enhance the resuscitation capabilities of *BPW*, such as an iron source, sodium pyruvate and sodium dexoycholate. Some studies reported a better recovery of stressed STEC cells with these supplements, while others observed the opposite (Margot *et al.*, 2015; Weber *et al.*, 2009). Since STEC is a fast grower, it has also been suggested that enrichment in a non-selective medium, such as *BPW*, may be an option without the risk of overgrowth by the background microbiota (Jasson *et al.*, 2009). However, the composition of the background microbiota is important. For the recovery of *E. coli* O157 from sprouted seeds, a matrix containing high levels of competing microbiota, the best results were achieved after enrichment in *BPW* supplemented with a combination of three antibiotic (acriflavin (10 mgL<sup>-1</sup>), cefsulodin (10 mgL<sup>-1</sup>) and vancomycin (8 mgL<sup>-1</sup>)) (Weagant and Bound, 2001). Moreover, its use allowed for the detection of a wide range of non-O157 STEC strains, however, only when incubated at 37°C. While, 40,2°C and 41,2°C were reported to be the optimum incubation temperature for *E. coli* O157 and non-O157, respectively, the combination of these antibiotics and an incubation temperature of 42°C proved unsuitable for a number of STEC strains (Baylis, 2008; Chapman *et al.*, 2001; Gonthier *et al.*, 2001). On the other hand, a non-selective enrichment incubated at a higher temperature may be preferable to the use of antibiotics for the detection of some STEC strains (Kanki *et al.*, 2011).

#### 4.4.2 Antimicrobial components

A multitude of antimicrobial components have been used in the STEC detection and isolation protocols, either alone or in combinations. However, the type of antibiotic and the concentration has a major impact on the detection and isolation efficiency.

A commonly used selective component is tellurite (TeO<sub>3</sub><sup>2-</sup>), the toxic form of the metalloid Tellenium (Te<sup>0</sup>). It is toxic for most organisms, even at a low concentration. While, the exact

mechanism of its toxicity is still poorly understood, Gram-positive bacteria seemed to be more resistant compared to Gram-negative bacteria. Whereas little is known about these resistance mechanisms as well, several genetic tellurite resistance determinants have been characterized in Gram-negative bacteria, most of which are located on plasmids (Charsteen *et al.*, 2009). Some of the *E. coli* strains are known to possess such genes located in a *ter* operon (Orth *et al.*, 2007). The minimum inhibitory concentration (MIC) of tellurite for a broad range for STEC strains seemed to vary between 2 to 12,5 mgL<sup>-1</sup> (Fukushima *et al.*, 2000; Hiramatsu *et al.*, 2002; Zelyas *et al.*, 2016). However, most sorbitol fermenting *E. coli* O157:NM and *E. coli* O103:H2 lack these *ter* genes, in addition to some strains belonging to other STEC serotypes. Therefore, these strains will not grow in media containing tellurite (Beutin and Fach, 2014; Orth *et al.*, 2007).

Novobiocin is an aminocoumarin, the simplest natural product of Streptomyces, which shows low penetration into Gram-negative bacteria, but is effective against Gram-positive bacteria (Lawson and Stevenson, 2012). However, some Gram-negative bacteria, including some STEC strains, have been reported to be sensitive to novobiocin. Although, the ISO recommend the use of 20 mgL<sup>-1</sup> for the detection of *E. coli* O157:H7; the MIC value of non-O157 STEC strains was determined at 16 mgL<sup>-1</sup> (ISO, 2001; Vimont *et al.*, 2007). Therefore, the concentration of novobiocin was reduced to 16 mgL<sup>-1</sup> in the enrichment protocol of ISO/TS 13136:2012 (Figure I-5) (ISO, 2012a).

Other antibiotics, such as cefixime, vancomycin, cefsulodin, acriflavin are less frequently used for their ability to suppress the growth of Gram-positive bacteria. In addition, cefsulodin is known to inhibit *Pseudomonas*, *Aeromonas* spp. and coliforms (Hussein *et al.*, 2008). Acriflavin is recommended by the ISO for the detection of both *E. coli* O157 and non-O157 STEC serogroups from dairy products in which the background microbiota may be very important (ISO, 2012a, 2001; Savoye *et al.*, 2011).

#### 4.4.3 Stressed STEC

It should also be taken into account that STEC cells are often impaired by sub-lethal injuries or stress sustained during food processing and storage (e.g. exposure to salt, acid, cold, heat, freeze stress). While, these cells remain viable and capable to cause illness, they may not be culturable. Therefore the detection and isolation of stressed STEC in food by either a selective enrichment step or direct plating on a selective agar may lead to underestimations (De Boer and Heuvelink, 2000; Smith and Fratamico, 2012; Stephens and Joynson, 1998).

The acid tolerance of STEC was already mentioned, as it is essential to pass the gastric environment and colonize the intestines. Therefore, STEC is able to survive in acidic food, such

as dairy products, mayonnaise, apple cider and fermented products. While, a clear difference in survival rates at a low pH can be observed between STEC strains, no correlations can be made between certain serotypes or source of isolation (Benjamin and Datta, 1995). However, exposure to mild acidity can induce a higher acid tolerance within the bacteria. Therefore, the STEC transmitted to human through acidic food might be more able to pass the extreme gastric acidity (Bergholz and Whittam, 2007).

Outbreaks of STEC have been attributed to the consumption of frozen products, implicating that the freezing process was insufficient to succumb all pathogens (Archer, 2004). Forty four hours at -23°C did result in a significant decrease in STEC numbers, but enough remained to cause potential illness. (Dykes, 2006). Besides freezing, food is often kept at refrigerator temperature to prolong shelf life. STEC was reported to survive for 18 days in yoghurt, 30 days for orange juice kept at 4°C and 11 days in ground meat kept at 7°C (Duffy *et al.*, 2006; M. Uyttendaele *et al.*, 2001). It is generally observed that the survival rates are even higher when acid and cold stress are combined (Elhanafi *et al.*, 2004). As the bacterial metabolism varies with the different growth phases, the highest acid tolerance was observed during the stationary phase. In accordance, *E. coli* is much more tolerant to freeze injury during the stationary growth phase compared to exponential phase (Archer, 2004; Benjamin and Datta, 1995).

A low water activity ( $a_w$ ) is also often employed to inhibit the bacterial growth. However, in accordance with *Salmonella* spp., STEC exhibits a remarkable dryness tolerance. STEC was able to survive for 35 to 70 days at an  $a_w$  value of 0,56 stored at 25°C and up until two years when stored at 4°C. However, STEC died within 24 hours when this low  $a_w$  was combined with a pH below 4 (Hiramatsu *et al.*, 2005).

#### 4.4.4 Cultural isolation

The cultural isolation, using solid selective agar plates, remains the only tool to purify the organism for further characterization. This process has proven to be the most laborious part of the STEC screening (Bettelheim and Beutin, 2003).

Cultural isolation as a means to detect and isolate STEC has been carried out routinely for *E. coli* O157:H7. In order to identify sorbitol-negative *E. coli*, sorbitol in a common fermentation medium is used, namely Sorbitol-MacConkey agar (SMAC). This medium contains sorbitol instead of lactose, because these *E. coli* are unable to ferment sorbitol. Therefore no shift in pH will occur and the respective colonies will remain colorless (Deisingh and Thompson, 2004; March and Ratnam, 1986). However, other sorbitol-negative bacteria, such as *Proteus* spp. and some *E. coli*, grew indistinct from *E. coli* O157:H7 on this agar. Therefore, rhamnose and cefixime (0,05 mgL<sup>-1</sup>)

was added to the agar, because only *E. coli* O157:H7 is unable to ferment rhamnose and the other sorbitol-negative bacteria are inhibited by cefixime (Chapman *et al.*, 1991). Later on, rhamnose was replaced by potassium tellurite (2,5 mgL<sup>-1</sup>) because of its inhibitory activity against *E. coli* strains other than *E. coli* O157:H7. This CT-SMAC agar medium is still recommended for the detection of *E. coli* O157 (ISO, 2001).

Besides the inability to ferment sorbitol, *E. coli* O157:H7 typically lacks  $\beta$ -glucuronidase activity (GUD). To enhance the specificity of the agar media a number of chromogenic substrates have been developed. Due to the lack of GUD, *E. coli* O157:H7 is unable to cleave a colourless fluorogenic substrate 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) to the fluorescent end product, methylumbelliferone, which is visible under UV light (Hussein and Bollinger, 2008). However, this fluorescent end product was often observed to diffuse into the agar, making the *E. coli* O157 harder to distinguish. Therefore, a chromogenic substrate 5-bromo-4-chloro-3-indoxyl- $\beta$ -D-glucuronide (X-Gluc) was developed that showed a blue colony colour after cleavage and no diffusion (Okrend *et al.*, 1990). On the other hand, most *E. coli*, including *E. coli* O157:H7, possess the  $\beta$ -galactosidase enzyme able to cleave lactose into glucose and galactose. Therefore, many agar media contain the colourless chromogenic substrate 5-bromo-4-chloro-3-indoxyl- $\beta$ -D-galactopyranoside (X-gal) which results in a blue-green colony colour after cleavage. Each chromogenic substrate can be substituted by different halogens, resulting in a variety of colony colours (Druggan and Iversen, 2014; Eden, 2014). These biochemical features were the basis of many commercial chromogenic agars developed ever since, such as Rainbow® Agar O157 (Biolog) and CHROMagar™ O157 (CHROMagar) (Table I-3). However, the inability to ferment sorbitol and lack of GUD is not linked to the Stx production and proved useless for the detection of non-O157 STEC serotypes and even for other sorbitol fermenting H-types of the O157 STEC serogroup (Nataro and Kaper, 1998). On the other hand, some non-O157 STEC strains were reported to share these biochemical features. Hence, these features are not exclusive for *E. coli* O157:H7 (Hussein *et al.*, 2008). However, no such biochemical markers have been described to distinguish all STEC from other bacteria and especially other Enterobacteriaceae. Therefore, the detection of non-O157 STEC strains should preferably be carried out by the direct or indirect screening of Stx production after enrichment, using the methods mentioned above. Only those samples that test positive during the STEC detection methods, should be subjected to an isolation step, using suitable isolation agar media (Bettelheim and Beutin, 2003; He *et al.*, 2011). The use of washed sheep blood agar was proposed. Since STEC seemed to produce a zone of hemolysis on this agar. However, not all STEC produce enough enterohemolysin necessary for the formation of such a zone. Therefore, mitomycin C was sometimes added to induce the production of the hemolysin. Still, only 40% of all STEC strains produce this hemolysin, in contrast to 90% of EHEC strains.

Moreover, some EPEC and commensal *E. coli* strains have been reported to produce hemolysin as well (Beutin and Fach, 2014; Sugiyama *et al.*, 2001).

In the past decade, a number of chromogenic and non-chromogenic isolation media have been developed to identify *E. coli* O157 and/or non-O157 STEC strains by observing colony growth, morphology and color. Many of these media employ tellurite. But as mentioned above, a number of STEC strains are sensitive to this antibiotic agent and will not grow on these agar plates and might be overlooked (Jinneman *et al.*, 2012; Possé *et al.*, 2008; Tzschoppe *et al.*, 2012).

#### 4.4.5 Combination assay: immune-capture and culture

Because the present enrichment medium and isolation agar are still not suitable for the detection and isolation of all STEC strains, immunomagnetic separation (IMS) can be employed, either on the sample directly, or on the enriched sample. This method uses antibody-coated magnetic beads that will bind the target organisms. The formed complexes can be separated from the sample using an external magnetic source. The separated organisms are plated on an isolation medium. The sensitivity will increase, because this inoculum has been concentrated relative to the background microbiota. However, the presence of the high level of background microbiota may lead to carry-over of non-target organisms and again decrease the sensitivity (Chapman *et al.*, 2001; Deisingh and Thompson, 2004; Nou *et al.*, 2006). The use of IMS is recommended by the ISO for the detection of *E. coli* O157:H7 from food (ISO, 2001). However, the isolation of the non-O157 STEC strains were not improved with IMS, partly due to loss-making factors resulting in too weak antibody-antigen complexes (Verstraete *et al.*, 2010). Moreover, only a limited number of IMS beads directed to the most common non-O157 STEC serogroups are commercially available (Beutin and Fach, 2014).

An acid treatment has been proposed to eliminate these carry-over microbiota from the IMS beads (Fedio *et al.*, 2012; Yoshitomi *et al.*, 2012). This technique exploits the acid tolerance of *E. coli* to reduce the level of background microbiota. Acid treatment prior to plating on selective isolation media might be a rapid and economical alternative way to isolate STEC, especially for STEC serogroups without commercially available IMS beads (Fedio *et al.*, 2012; Grant *et al.*, 2009; Tillman *et al.*, 2012).

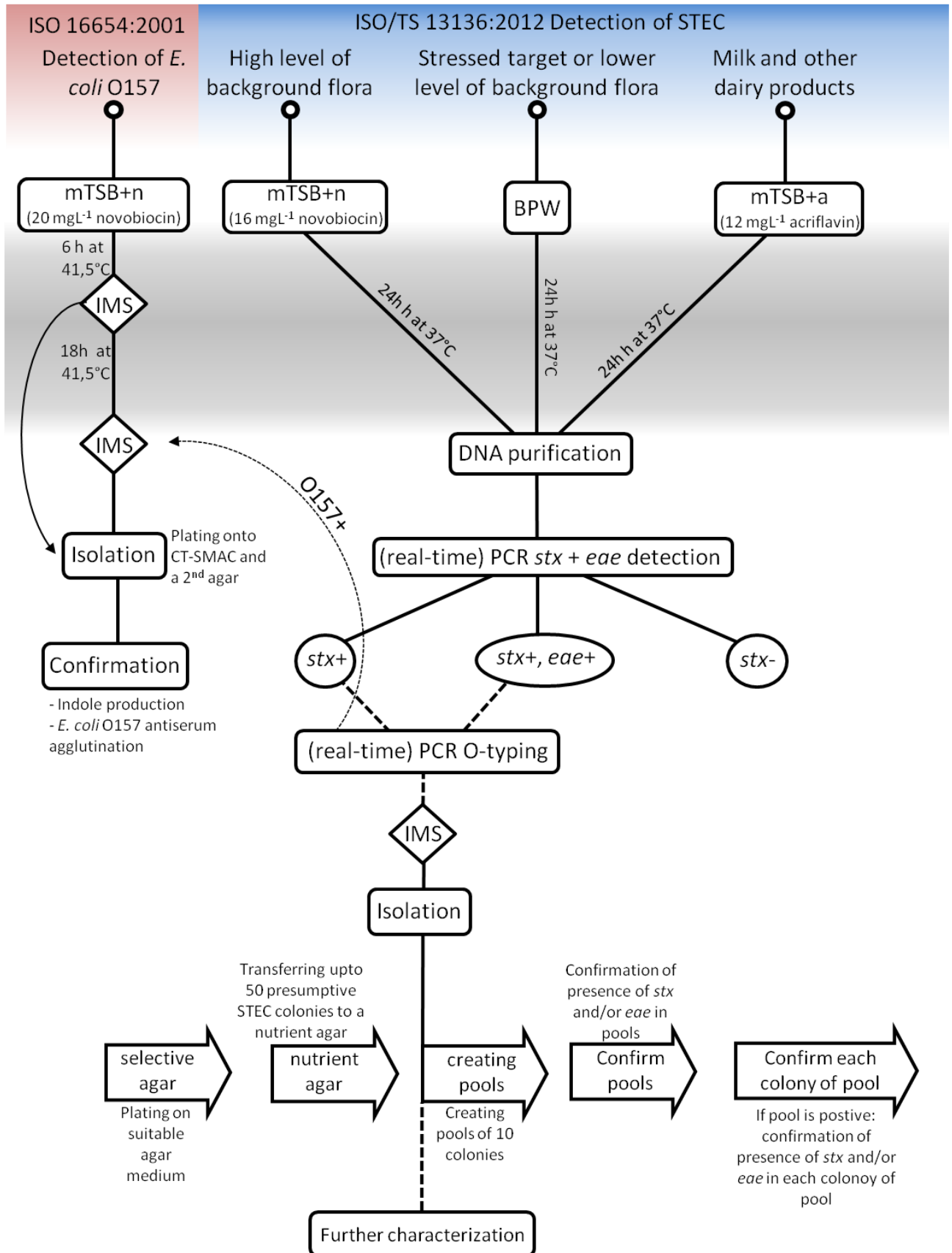


Figure I-5. Flow chart illustrating the detection and isolation of STEC from food and animal feeding stuffs as recommended by the ISO/TS 13136:2012 and *E. coli* O157 as recommended by the ISO 16654:2001 (ISO, 2012a, 2001). All dotted lines indicate facultative steps.



Table 1-3.. Appearance of *E. coli* O157, non-O157 STEC and other *E. coli* strains on commonly used and studied agar media.

Medium	Antibiotic component	<i>E. coli</i> O157	STEC non-O157	Other <i>E. coli</i>	Author
MacConkey		Fuchsia	Fuchsia	Fuchsia	(Gill <i>et al.</i> , 2014)
Sorbitol MacConkey		Colourless	Fuchsia	Fuchsia	(March and Ratnam, 1986)
CT-SMAC	Cefixime, Tellurite	Colorless	Fuchsia	Fuchsia	(Jinneman <i>et al.</i> , 2012)
CT-Rhamnose Mac	Cefixime, Tellurite	Green	O26: Dark-Blue/Green; O103,O111,O145:Green	Green	(Hara-Kudo <i>et al.</i> , 2002)
MUG Mac		Colorless + no fluorescence	Blue fluorescence		(Hussein and Bollinger, 2008)
CT-XGluc-MAC	Cefixime, Tellurite	Blue	Violet		(Tzschoppe <i>et al.</i> , 2012)
Washed sheep's blood agar <sup>1</sup>	Vancomycin, cefsulodin, cefixime	small turbid zone of hemolysis	small turbid zone of hemolysis	No hemolyse/small turbid zone of hemolysis	(Hornitzky <i>et al.</i> , 2001; Sugiyama <i>et al.</i> , 2001)
Levine Eosin Methylene Blue Agar		Green + dark centre	Green + dark centre	Green + dark centre/White	(Jinneman <i>et al.</i> , 2012; Kase <i>et al.</i> , 2015)
CT-TBA	Cefixime, Tellurite	White	White/pinpoint		(Gill <i>et al.</i> , 2014)
Eosin Methylene Blue-TBA	Cefixime, Tellurite	Pink	Pink/mauve/pin point		(Gill <i>et al.</i> , 2014)
Tryptone Bile X-Glucuronide		White/Blue-Green	Blue-Green	Blue-Green	(Tzschoppe <i>et al.</i> , 2012)
Possé O157	Novobiocin, Tellurite	Sor+: Purple; Sor-: Blue-Green			(Possé <i>et al.</i> , 2008)
Possé non-O157	Novobiocin, Tellurite		O26: Purple; O103, O111: Blue; O145: Green; all with dark centres		(Possé <i>et al.</i> , 2008)
VTEC agar	Vancomycin, Cefsulodin	Pink + no fluorescence	Mauve/pink/white +/- fluorescence	Mauve/Pink/Colourless	(Gill <i>et al.</i> , 2014, 2012)

USMARC agar	Novobiocin, Tellurite	Green/Blue-Green	O26: Blue; O103: Blue-Green/Green; O111: Dark-Blue/Green; O145: Purple	Purple/Blue-grey/Blue-green	(Kaichayanand <i>et al.</i> , 2013)
SHIBAM	Mitomycin C	Grey + hemolyse	Grey +/- hemolysis	Grey + no (weak) hemolysis	(Kase <i>et al.</i> , 2015)
XM-EHEC (Nissui Pharma. Co. Ltd., Tokyo, Japan)	Unk	Magenta	O26, O103, O145: blue; O111: magenta	Magenta, Colourless	(Teramura <i>et al.</i> , 2013)
EHEC-chrom	Cefixime, Tellurite	Blue-Green	Blue-green	Colourless	(Teramura <i>et al.</i> , 2013)
Rainbow agar® O157 – USDA (Biolog, Hayward, CA)	Novobiocin, Cefixime, Tellurite	Blue-Grey/Purple	O26: Mauve/Pink; O103, O111: Grey/Mauve/Blue/Pink/White; O145: Grey/Mauve/Blue	Pink/Mauve	(Gill <i>et al.</i> , 2014; Kase <i>et al.</i> , 2015)
Rainbow agar® O157 – USDA + WSB (Biolog)	Novobiocin, Cefixime, Tellurite	Mauve/Blue-Grey + hemolysis	O26: Mauve/Grey + hemolysis; O103, O145: Mauve + hemolysis, O111: Blue/Mauve + hemolysis	Pink/Mauve +/- hemolysis	(Kase <i>et al.</i> , 2015)
Rainbow agar® O157 – low background (Biolog)	Novobiocin, Tellurite	Blue-Grey	O26: Mauve/Blue-Grey; O103: Grey/Mauve/Blue/ White; O111: Grey/Blue/White, O145: Grey/Mauve/ Blue	Mauve/Pink/Blue/White	(Gill <i>et al.</i> , 2014; Jinneman <i>et al.</i> , 2012)
R&F <i>E. coli</i> O157:H7 (R&F Laboratories, Downers Grove, IL)	Unk	Blue-Black	Black to dark green	Green/yellow/Blue/White	(Kase <i>et al.</i> , 2012)
CHROMagar™ <i>E. coli</i> (CHROMagar, Paris, France)	Unk	Sor+: White, Sor-: Blue	blue	Blue	(Tzschoppe <i>et al.</i> , 2012)
CHROMagar™ O157 (CHROMagar)	Unk	Mauve with dark centre	Blue-white/white	Blue /White/pin point	(Jinneman <i>et al.</i> , 2012)
CHROMagar™ O26/O157 (CHROMagar)	Unk	Red	O26: Green, O103, O111, O145: Violet	Violet	(Tzschoppe <i>et al.</i> , 2012)
CHROMagar™ STEC (CHROMagar)	Unk	Mauve/Pink + no fluorescence	Mauve/Pink + fluorescent	Yellow/White	(Tzschoppe <i>et al.</i> , 2012)
CHROMagar™ STEC-WSB (CHROMagar)	Unk	Mauve/pink	Mauve+/- hemolysis	Mauve/Grey/Blue/Colourless	(Kase <i>et al.</i> , 2012)
O157:H7 ID agar (bioMérieux SA, Marcy-l'Etoile, France)	Unk	Blue-Green	Green	Purple/Red/Colourless	(Bettelheim, 2005)

TBA Tryptone Bile agar; USMARC: U.S. Meat Animal Research Center, SHIBAM: STEC Heart Infusion Blood Agar, WSB: Washed sheep's blood

<sup>1</sup> sometimes supplemented with mitomycin C, \* strains reported with no growth on agar medium

Sor+: sorbitol fermenting strain; Sor-: non-sorbitol fermenting strain; unk: commercial media with unknown composition

## 5. *Characterisation of STEC*

Once a STEC strain is isolated it can be further characterized in order to differentiate the STEC strains below species level. A number of microbiological and molecular methods have been described for this purpose. These methods are mainly used to investigate transmission routes, identify the source of a foodborne outbreak and molecular risk assessment to determine the public health impact. Secondly, they are used for population genetics to assess the diversity and evolutionary relationships within the STEC population (Karama and Gyles, 2010).

### 5.1. *Serotyping*

The use of serotyping is limited in routine diagnostic laboratories, since most are not equipped to perform serotyping for the more than 400 different described STEC serotypes. Therefore, they will focus on the most common STEC serotypes. For these serotypes a number of commercial immunological tests are available, using agglutination assays as described above (4.2 Immunological assays). However, as more STEC serotypes are being isolated from humans, food or other sources, many appear to be untypeable for the O- and H-antigen due to cross-reaction between serogroups or the presence of O-rough strains. These O-rough strains lack the O-chains in the lipopolysaccharide, leading to autoagglutination. Much of these difficulties derive from extensive genetic variation within the *rfb* gene cluster encoding the O-antigen. Therefore, a DNA-based approach is faster, less expensive and more specific compared to these immunological tests. The *rfb* gene cluster, flanked by the conserved JUMPstart sequence and a housekeeping gene *gnd*, contains several genes, such as *wzx* and *wzy*. Since, the latter two genes are very distinct among different serogroups, they are often targeted by primers used in serogroup-specific PCR assays. (Sánchez *et al.*, 2015; Wang and Reeves, 1998). The flagella (H-antigen) is encoded and associated with more than 40 genes, mostly located in four gene clusters. The *fliC* cluster encodes most of the flagellar antigens in *E. coli* (Wang *et al.*, 2003). PCR assays with primers targeting these genes were able to identify the H-type of many phenotypical non-motile (NM) STEC strains.

A number of other DNA-based serotyping methods have been described. Many bacteria, including *E. coli*, possess clustered, regulatory interspaced, short palindromic repeats (CRISPR) within its genome. The CRISPR locus is closely related to certain *E. coli* O:H antigens and virulence factors. Therefore, a real-time PCR assay was developed to target genetic markers within the CRISPR locus specific for STEC serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28 and O157:H7 and strongly correlated with STEC virulence factors (*stx* and *eae* genes) (Delannoy *et al.*, 2012). Another approach was based on the sequencing results of the conserved *gnd* gene flanking the

O-antigen gene cluster. All *gnd* genes appeared to be identical within the same STEC serogroup and distinct between different STEC serogroup. Moreover, the *gnd* genes were distinct between STEC and non-STEC strains belonging to the same serogroups. This approach may differentiate STEC from non-STEC serogroups (Gilmour *et al.*, 2007). Furthermore, certain single nucleotide polymorphisms (SNPs) were identified within the O-antigen gene cluster that differentiate between the six most common serogroups (O26, O45, O103, O111, O121, O145) and are associated with STEC strains (Norman *et al.*, 2012). These new methods of serotyping may lead to novel culture-independent detection methods for STEC.

## 5.2. Genotyping

### 5.2.1 Pulsed-Field Gel Electrophoresis (PFGE) and multilocus sequence typing (MLST)

PFGE is often employed to characterize O157:H7 STEC strains and more recently for the non-O157 STEC strains. Because of its high discrimination power, PFGE remains the ‘gold standard’ of the subtyping methods. While, it is time consuming in regard to sample preparation and running time, it is highly standardized for inter and intra-laboratory comparisons (Anderson *et al.*, 2015). The organism is embedded in agarose, in which it is lysed and its intact genome cleaved into multiple fragments (15 to 1130 kb) by one or more restriction enzymes. For STEC, primary enzyme *XbaI* is used for screening of relatedness. A secondary (*BlnI*) and third (*SpeI*) enzyme can be used for confirmation of the obtained results, especially in case of identical patterns. After a specific electrophoresis, a pattern of ten to 25 bands can be visualized (Gerner-Smidt *et al.*, 2006). In theory the patterns of the same strain isolated throughout an outbreak should be identical. However, random genetic events over time may result in a slightly altered pattern, making the interpretation more difficult. Because no consensus is formulated by PulseNet to standardize the decision on the relatedness of isolates, the comparison of patterns remains, in part, a subjective process (Tenover *et al.*, 1995). However, the specialized software-based similarity coefficients may give a good indication of genetic relatedness between isolates (Karama and Gyles, 2010). Another commonly used characterisation method is MLST. MLST categorizes isolates per sequence type (ST) derived from the detected variations in internal fragments of seven housekeeping genes. However, it lacks the discriminatory power of PFGE, because distinct isolates, determined by PFGE, are often annotated to an identical ST (Karama and Gyles, 2010). However, MLST played an important role in the investigation of the origin of pathogenic *E. coli* and evolutionary relationships within the *E. coli* population (Leopold *et al.*, 2009).

### 5.2.2 Phage typing

Since the first outbreak of *E. coli* O157:H7 in 1982 an exponential rise in the number of human isolates of *E. coli* O157:H7 has been observed. In order to allow further differentiation within this serotype phage typing was performed. According to a scheme a number of bacteriophages are used that produce a phage infection profile of the *E. coli* O157:H7 strain based on the level of lysis achieved by each phage (Cowley *et al.*, 2015; Khakhria *et al.*, 1990). Over 88 different phage types (PT) have been described for STEC O157:H7. While some PT's appear more likely to be associated with human infection, so far still little is known of the basis for this (Mora *et al.*, 2004). Phage typing is dependent upon the phage constitution or insertion history of the strain, because the presence of a different phage or a phage remnant can provide resistance to infection with other phages. Furthermore, super-shedding in cattle might be associated with the infection of a particular *E. coli* O157 phage type, such as PT21/28 (Chase-Topping *et al.*, 2008). However, for epidemiological and outbreak investigation the level of discrimination is insufficient. Therefore, phage typing is often used in combination with other typing techniques, such as PFGE (Mora *et al.*, 2004; Rivas *et al.*, 2006).

### 5.2.3 Phylogenetic group

*E. coli* is composed of four phylogenetic groups (A, B1, B2 and D). The extra-intestinal *E. coli* belongs mostly to group B2 and, in a lesser extent, to group D and most commensal *E. coli* fall into group A. The phylogenetic analysis is performed by amplifying three marker genes (*chuA*, *yjaA* and *tspE4.C2*) using a multiplex PCR (Clermont *et al.*, 2000). STEC strains are distributed over all four groups, but segregate mainly in B1 and rarely belong to B2. Most of the HUS-associated STEC strains belong to group B1 and to a lesser extent to group D. While, group A was long considered to harbour less-virulent *E. coli* strains, a significant number of STEC strains fell into this group (Chaudhuri and Henderson, 2012; Franz *et al.*, 2015; Girardeau *et al.*, 2005).

### 5.2.4 Virulence profile

Because, the detection of STEC is mainly based on the either direct or indirect detection of Stx, many STEC strains will be identified that do not cause any clinical symptoms. Therefore, assessing the clinical and public health risks associated with different STEC strains remains of utmost importance (Karmali *et al.*, 2003).

In the past years, subtyping of the Stx toxin has become more important to describe potential human pathogenic STEC strains. Stx1 and Stx2 can be divided in three and seven subtypes, respectively. Moreover, at present a total of 107 Stx variants have been described within the different Stx subtypes: 9 variants of Stx1a (including Stx from *Shigella dysenteriae*), 4 of Stx1c, 1 of

Stx1d, 21 for Stx2a, 16 for Stx2b, 18 for Stx2c, 18 for Stx2d, 14 for Stx2e, 2 for Stx2f and 4 for Stx2g. A Stx variant was defined by one or more amino acid difference in the analyzed sequences compared to the other sequences. The Stx subtype, and maybe the variant, have been linked to the severity of human illness (Scheutz, 2014; Scheutz *et al.*, 2012). Stx2e is mostly isolated from swine and food of porcine origin. While, this toxin plays a major role in the porcine edema disease, it is seldom associated with human disease (Kaufmann *et al.*, 2006). Stx2f is the latest discovered subtype and is mainly found in birds. However, a few cases of human illness have been reported caused by this subtype. Still, the illness was less severe compared to O157 STEC infections (Friesema *et al.*, 2014). Stx1c and Stx2b are associated with diarrhea in humans, however, rarely developing to bloody diarrhea or HUS. These two subtypes are most frequently found in small ruminants and their food products (Brett *et al.*, 2003). On the other hand, Stx1a, Stx2a, Stx2c and Stx2d are frequently found in bovines and their food products and associated with bloody diarrhea and HUS. Stx2a is highly associated with HUS (e.g. the highly virulent outbreak strain O104:H4 STEC). *In vitro* studies on Vero cells and human renal cells showed that Stx2a and Stx2d were at least 25 times more potent than Stx2b and Stx2c. The latter two have similar potency as Stx1 (Beutin and Fach, 2014; Fuller *et al.*, 2011; Scheutz, 2014).

Besides the *stx* genes, other virulence markers have been described, such as the *eae* gene and the EHEC-*hlyA/ehxA* gene. As mentioned above, the term “enterohemorrhagic *E. coli*” (EHEC) was devised to denote this particular subgroup of pathogenic STEC strains carrying these genes. However recently, many more virulence and adherence factors have been identified that are involved in the pathogenicity of STEC (Karmali *et al.*, 2003; Scheutz, 2014). These factors are most often encoded by genes located on mobile genetic elements, such as plasmids, bacteriophages, transposons and pathogenicity islands (PAI).

The characterization of these genes has led to a novel approach in the identification of virulent STEC strains, namely the “Molecular Risk Assessment” (MRA) approach. This MRA approach was first established by Karmali *et al.* (2003) by classifying the different STEC strains in seropathotypes according to its pathogenicity for humans. Later studies attempted to further specify the MRA approach by linking the presence of described virulence genes to severe illness and outbreaks. The genes located on the different PAI were the main focus in many of these studies (Table I-4). The PAI is a class of genomic islands present on the genomes of pathogenic strains, but absent on the genomes of non-pathogenic members of the same or related species (Hacker and Kaper, 2010). These islands, distributed throughout the genome as O-islands (OI), contain a flexible gene pool contributing to pathogen evolution and virulence potential. The best known PAI for STEC is the LEE, which harbors the *eae* gene. In recent years many non-LEE-encoded effector (NLE) genes were identified. Although, the contribution of these genes to the

virulence of the different STEC strains is mainly unknown, these NLE genes harbored on OI-122 (e.g. *ent/espL2*, *nleB*, *nleE*) and on OI-71 (e.g. *nleA*, *nleF*, *nleH1-2*) were most frequently present in STEC strains associated with severe disease in humans (Bugarel *et al.*, 2011). Other PAI have been described, such as OI-57, OI-43/48 and OI-36. However, disease-associated STEC strains have been reported that harbor only a few NLE genes. Therefore, other virulence factors remain of crucial importance. (Coombes *et al.*, 2008; Ju *et al.*, 2014). Furthermore, plasmids may carry additional virulence and adherence factors such as enterohemolysin (*ehxA*), STEC auto-agglutinating adhesion (*saa*), subtilase cytotoxin (*subA*) and catalase-peroxidase (*katP*) (Brunner *et al.*, 1999; Paton and Paton, 2002; Paton *et al.*, 2004).

Table I-4. Overview of the main virulence factors of STEC (Berenger *et al.*, 2015; Bolton, 2011; Coombes *et al.*, 2008; Franz *et al.*, 2015; Monaghan *et al.*, 2012).

Gene	Genetic support	Encoded protein or family effector
<i>stx1</i>	chromosome	Shiga toxin
<i>stx2</i>	chromosome	Shiga toxin
<i>lpf</i>	chromosome	long polar fimbriae
<i>chuA</i> <sup>1</sup>	chromosome	hemoglobin receptor
<i>tspE4, C2</i> <sup>1</sup>	chromosome	esterase-lipase protein
<i>yjaA</i> <sup>1</sup>	chromosome	unknown
<i>rpoS</i>	chromosome	RNA polymerase
<i>eae</i>	LEE	intimin adhesin
<i>tir</i>	LEE	translocated intimin receptor
<i>cesT</i>	LEE	molecular chaperon for tir
<i>espA</i>	LEE	effector proteins secreted by T3SS
<i>espB</i>	LEE	effector proteins secreted by T3SS
<i>espD</i>	LEE	effector proteins secreted by T3SS
<i>tir/espE</i>	LEE	effector proteins secreted by T3SS
<i>map</i>	LEE	effector proteins secreted by T3SS
<i>espF</i>	LEE	effector proteins secreted by T3SS
<i>espG</i>	LEE	effector proteins secreted by T3SS
<i>espH</i>	LEE	effector proteins secreted by T3SS
<i>ent/espL2</i>	OI-122	F-actin aggregation/microcolony formation
<i>nleB</i>	OI-122	Immunomodulation, type III effector
<i>nleE</i>	OI-122	immunomodulation
<i>efa 1</i>	OI-122	EHEC factor for adherence
<i>pagC</i>	OI-122	PagC-like membrane protein
<i>efa 2</i>	OI-122	EHEC factor for adherence
<i>nleB2</i>	OI-36	type III effector
<i>nleC</i>	OI-36	immunomodulation, zinc-metalloprotease
<i>nleH1-1</i>	OI-36	immunomodulation
<i>nleD</i>	OI-36	immunomodulation, zinc-metalloprotease
<i>iha</i>	OI-43/OI-48	iron-regulated gene A homolog adhesin
<i>terB</i>	OI-43/OI-48	tellurite resistance cluster
<i>ureC</i>	OI-43/OI-48	urease-associated protein
<i>nleG2-3</i>	OI-57	ubiquitin ligase
<i>nleG6-2</i>	OI-57	ubiquitin ligase
<i>nleG5-2</i>	OI-57	ubiquitin ligase
<i>ckf</i>	OI-57	killer protein
<i>nleG</i>	OI-71	ubiquitin ligase
<i>nleF</i>	OI-71	disruption protein trafficking, type III effector
<i>nleH1-2</i>	OI-71	Immunomodulation, type III effector
<i>nleA</i>	OI-71	disruption tight junctions and protein trafficking, type III
<i>nleG2-1</i>	OI-71	ubiquitin ligase
<i>nleG9</i>	OI-71	ubiquitin ligase
<i>ecs1822</i>	OI-71	Hypothetical protein
<i>espM1</i>	OI-71	Type III effector
<i>astA</i>	plasmid/chrom	EAggEC HS enterotoxin EAST 1
<i>saa</i>	pO113	STEC autoagglutinating adhesion
<i>sab</i>	pO113	STEC autotransporter
<i>subA</i>	pO113	subtilase cytotoxin
<i>eibG</i>	pO113	Immunomodulation
<i>EHEC-hlyA/ehxA</i>	pO157	enterohemolysin, pore-forming cytolysin
<i>katP</i>	pO157	catalase peroxidase
<i>etpD</i>	pO157	type II secretion effector protein
<i>espP</i>	pO157	extracellular serine protease autotransporter
<i>toxB</i>	pO157	adhesion, homolog to efa1
<i>ompA</i>	pO157	Outer membrane protein A, adhesin
<i>stcE</i>	pO157	Esterase inhibitor, type II effector

<sup>1</sup> genes amplified in the phylogenetic group PCR



**Aims**

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Shiga toxin-producing *Escherichia coli* (STEC) belongs to the top five of the most frequently reported zoonosis in Belgium, and the majority of cases is caused by STEC O157:H7. Ruminants, especially cattle, are colonized by STEC and are regarded as the natural reservoir. Although animals infected with STEC normally show no signs of disease, it can be very pathogenic to humans, causing mild to severe clinical symptoms. STEC can be transmitted to humans through many different routes, but mainly through consumption of contaminated foods. The development of a detection and isolation method has long been targeting STEC O157:H7, resulting in the widely accepted International Organization for Standardization (ISO) standard 16654:2001. However, non-O157 STEC strains are being increasingly recognized and reported as important foodborne pathogens. Especially after the so-called “EHEC crisis” in Europe in 2011 the global awareness concerning this pathogen has intensified. However, the abovementioned method for the detection and isolation of *E. coli* O157:H7 fails to detect these serogroups. A new method was formulated in current ISO/TS 13136:2012 for the detection of the five most common STEC serogroups O26, O103, O111, O145 and O157 from food. However, the follow-up culture-based isolation of these strains remains problematic. Therefore, we aimed to optimize the strategy for the detection and isolation of non-O157 STEC from a broad variety of food matrices. Since, both low-pathogenic and highly pathogenic STEC strains produce Stx and are frequently recovered from food, assessing the pathogenic potential of these strains remains also of utmost importance. Finally, the accurate measurement of the concentration of shed STEC in cattle faeces could be a key answer to questions concerning transmission of STEC and contamination sources.

Therefore, the specific aims of this PhD-thesis were:

- The evaluation of different media used for the enrichment of non-O157 STEC strains, with regard to the resuscitation capabilities of sub-lethally injured STEC cells (*chapter 2*).
- The evaluation of different media used for the isolation of non-O157 STEC strains, with regard to their ability to support growth of *stx*-positive and *stx*-negative *E. coli* strains and the discriminative power based on colony morphology (*chapter 3*).
- The evaluation of the selected media in *chapter 2* and *3* for the detection and isolation of non-O157 STEC strains, using artificially contaminated food samples. In addition, the merit of the implementation of an acid treatment procedure was investigated, using both artificially and naturally contaminated food samples (*chapter 4*).
- The methodology for the detection of STEC described in *chapter 4* may increase the number of successfully isolated STEC strains. However, assessing the virulence potential of these strains remains crucial. Therefore, a collection of STEC strains isolated from food

and a number of STEC strains isolated from humans were characterized using PFGE and PCR followed by Luminex xMAP® in order to explore similarities between food and human isolates, and identify relevant virulence factors (*chapter 5*).

- Finally the novel droplet digital PCR (ddPCR) was compared to the features of the well-known quantitative real-time PCR (qPCR) for the quantification of STEC virulence genes *stx1*, *stx2* and *eae* in cattle feces (*chapter 6*). The potential advantages of this technique for the further enhancement methodology for the detection of STEC was discussed in *chapter 7*.

## **Chapter 2: Growth of stressed strains of four non-O157 Shiga toxin-Producing *Escherichia coli* serogroups in five enrichment broths**

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Adapted from:  
Verhaegen B., De Reu K., Heyndrickx M., Van Damme I., De Zutter L. (2015) Growth of Stressed Strains of Four Non-O157 Shiga Toxin-Producing *Escherichia coli* Serogroups in Five Enrichment Broths. Journal of Food Protection. Journal of Food Protection 78:11, 2012-2218

**Abstract**

The purpose of this study was to evaluate 1) the behavior of a number of strains of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serogroups (O26, O103, O111, O145) exposed to different stress conditions and 2) the growth dynamics of stressed and non-stressed non-O157 STEC cells in five enrichment media. STEC strains were exposed to acid, cold and freeze stress. Lethal and sub-lethal injury was determined by plating in parallel on selective and non-selective agar media. Freeze stress (8 days, -20°C) caused the most lethal ( $95.3 \pm 2.5\%$ ) injury, as well as the most sub-lethal ( $89.1 \pm 8.8\%$ ) injury in the surviving population. Growth of stressed and non-stressed pure cultures of non-O157 STEC on modified Tryptic Soy Broth (mTSB), Buffered Peptone Water (BPW), BPW with sodium pyruvate, Brila (Merck) and Stec Enrichment Broth (SEB; Bio-Rad) was determined using total viable counts. To compare growth capacities, the growth after seven and 24 hours enrichment was measured; lag phases and maximum growth rates were also calculated. In general, growth on BPW resulted in a short lag phase followed by a high maximum growth rate during the enrichment of all tested strains using all three stress types. Furthermore, BPW ensured the highest STEC count after seven hours of growth. Supplementing the medium with sodium pyruvate did not improve the growth dynamics. The two selective media, Brila and SEB, were less efficient than BPW but Brila's enrichment performance was remarkably better than SEB. This study shows that irrespective of the effect of background flora, BPW is still recommended for resuscitation of non-O157 STEC.

## 1. Introduction

*Escherichia coli* is present in the natural intestinal microbiota of mammals and in a variety of habitats, including soil, water, sediment and food. Within the species, some strains of *E. coli* can cause human diseases. The most severe of these are linked to strains belonging to the pathogenic group of the Shiga toxin-producing *E. coli* (STEC) (Catarama *et al.*, 2003; Tenaillon *et al.*, 2010). This group was first recognized in 1982 in patients with haemorrhagic colitis (HC) and was consequently named enterohaemorrhagic *E. coli* (EHEC) (Wells *et al.*, 1983). The causative agent responsible was STEC serotype O157:H7. This serotype is characterized by several virulence genes such as Shiga toxin genes, *stx 1* and *stx 2*, and the *eae* gene encoding an intimin. The symptoms range from non-bloody diarrhea, hemorrhagic colitis and haemolytic uraemic syndrome (HUS) to thrombotic thrombocytopenic purpura (TTP). STEC O157:H7 remains the most common serotype within the STEC group. The development of a detection and isolation method has therefore been targeted to this serotype, resulting in the widely accepted International Organization for Standardization (ISO) standard 16654:2001, which is based on the inability of most *E. coli* O157:H7 strains to ferment sorbitol (ISO, 2001). However, non-O157 STEC strains are being increasingly recognized and reported as important foodborne pathogens worldwide. In Europe, the most frequently isolated non-O157 STEC belong to the STEC serogroups O26, O91, O103, O111 and O145 (EFSA, 2013c; Johnson *et al.*, 1996; Mainil and Daube, 2005; Wang *et al.*, 2013). Because the abovementioned method for the detection and isolation of *E. coli* O157:H7 fails to detect these serogroups, a new method should be formulated in order to detect and isolate non-O157 STEC serogroups in a broad variety of matrices. In matrices such as foodstuffs, low numbers of STEC cells may have a large impact on public health and are often found in the presence of high numbers of closely related competitor organisms. Therefore, before any isolation step can be performed, a suitable initial enrichment step is required. This step should ensure the STEC cells' growth while inhibiting the competitor organisms. In addition, it should also be taken into account that STEC cells are often impaired by sub-lethal injuries sustained during food processing and storage (e.g. exposure to salt, acid, cold, heat, freeze stress). Despite the common occurrence of these stresses, little information is available concerning the growth of non-O157 STEC after exposure to the various physical and chemical stresses present in foods (Smith and Fratamico, 2012). A better understanding of the influence of stress types such as acid, cold and freeze stress on the growth of non-O157 STEC strains compared with non-stressed cells is needed. Furthermore, injured cells may become susceptible to selective agents included in enrichment media. Consequently, these media might be insufficient to resuscitate injured or stressed cells during the enrichment phase, possibly resulting in false negative results. The

selection of a suitable enrichment medium that facilitates the isolation and confirmation of STEC in foods is of great importance (Baylis, 2008; Catarama *et al.*, 2003; Jasson *et al.*, 2007).

A number of enrichment media are now commonly in use. The ISO recommends the use of modified Tryptone Soy Broth with the addition of novobiocin (16mg/l) or acriflavin (12mg/l) for the enrichment of food samples and dairy products, respectively (in TS 13136:2012). For the enrichment of stressed bacteria, buffered peptone water (BPW) is recommended (Catarama *et al.*, 2003; ISO, 2012a). While the ISO/TS specifies an enrichment period of 18 to 24 hours, multiple studies have proven the advantageous effect of a shorter enrichment period. This may be due to overgrowth of competing organisms and the release of PCR-inhibiting organic components during overnight incubation (Himathongkham *et al.*, 2007; Jasson *et al.*, 2009; Tzschoppe *et al.*, 2012).

The purpose of this study was to compare the growth dynamics of several pure cultures of non-O157 STEC strains in five enrichment media developed for the enrichment of members of the *Enterobacteriaceae*, including *E. coli* and coliforms, STEC and other Gram-negative pathogens from food samples. The following enrichment media were evaluated: mTSB as described by Doyle and Schoeni (1987), which differs from TSB by addition of bile salts and phosphate buffer; buffered Peptone Water (BPW) with and without the addition of sodium pyruvate (Weagant *et al.*, 2011); and two selective enrichment media, i.e. Brila and Stec Enrichment Broth (SEB).

In summary, the aims of the present study were (i) to determine the effect of acid, cold and freeze stress on strains belonging to non-O157 STEC serogroups and (ii) to compare the capabilities of five enrichment media to resuscitate these stressed bacteria.

## **2. Materials and methods**

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### **2.1. Strains**

Table II-1 lists the eight non-O157 STEC strains used in this study, namely two strains per serogroup: *E. coli* O26, O103, O111 and O145. These strains were all isolated from food samples and stored at -80°C using Pro-Lab Microbank cryovials (Pro-Lab, Ontario, Canada) according to the manufacturer's instructions. The strains were cultured onto Tryptone Soy Agar (TSA; Oxoid, Ltd., Basingstroke, Hampshire, England) at 37°C for 24h. These stock cultures were kept on TSA at 4°C and were renewed monthly. A single colony from these culture plates was transferred into Tryptone Soy Broth (TSB; Oxoid). After incubation at 37°C for 24h the stationary phase cells were ten-fold serially diluted in TSB to obtain a concentration of approximately 10<sup>6</sup> cfu/ml.



Table II-1. Overview of the non-O157 STEC strains. Swab: cattle carcass swab; milk: cow's milk.

Strain	Serogroup	Origin	Virulence genes			
			<i>eae</i>	<i>stx 1</i>	<i>stx 2</i>	<i>EHEC-hlyA</i>
MB 5948	O26	swab	+	-	+	+
MB 5316	O26	milk	+	+	-	+
MB 5307	O103	swab	+	+	-	+
MB 5308	O103	milk	+	+	-	+
MB 5949	O111	swab	+	-	+	+
MB 5310	O111	swab	+	+	+	+
MB 5305	O145	swab	+	+	-	+
MB 5850	O145	swab	+	-	+	+

## 2.2. Stress conditions

Acid, cold and freeze stress were provoked by creating conditions based on Jasson *et al.* (2007). Acid stress was effected by acidifying the inoculated TSB with HCl to a pH of 3.8, after which the broth was kept in the refrigerator at 4°C for 24 h. Cold and freeze stress were induced by storage of the inoculated TSB in a refrigerator at 4°C for seven days and in a freezer at -20°C for eight days, respectively.

## 2.3. Study of the behavior of non-O157 STEC subjected to stress conditions.

In the first part of the study the effect of acid, cold and freeze stress on non-O157 STEC serogroups was evaluated by calculating the percentage of lethal and sub-lethal injured cells. Initial counts before stress were determined by inoculating in parallel non-selective TSA and selective Tryptone Bile X-glucuronide (TBX; Oxoid) agar media using a spiral plater (Eddy Jet Spiral Plater, IUL instruments, Barcelona, Spain). Both agar media were incubated at 37°C for 24h and colonies were enumerated. The same procedure was performed after the different stress treatments and all experiments were repeated three times on different occasions for each individual strain.

A stressed or sub-lethally injured cell is defined as a cell that survives any lethal injury and is able to grow on a non-selective medium, but not on a selective medium (represented by TSA and TBX, respectively). The percentages of lethal and sub-lethal injured cells after the various stress treatments were calculated using the following formula (Besse *et al.*, 2000):

$$\% \text{ lethal injury} = \frac{\text{counts on TSA before stress} - \text{counts on TSA after stress}}{\text{counts on TSA before stress}} \times 100\%$$

$$\% \text{ sub - lethal injury} = \frac{\text{counts on TSA after stress} - \text{counts on TBX after stress}^*}{\text{counts on TSA after stress}} \times 100\%$$

\* TBX used as the selective medium for the enumeration of no sub-lethal injured cells

#### **2.4. *Growth in different enrichment media***

Five enrichment media were selected for the study: mTSB (modified TSB [Oxoid] buffered with 2.31 g/liter  $\text{KH}_2\text{PO}_4$  [Merck, KGaA, Darmstadt, Germany] and 11.04 g/liter  $\text{K}_2\text{HPO}_4$  [Merck]), BPW (Buffered Peptone Water [Bio-Rad, Marnes-la-Coquettes, France]), BPWp (Buffered Peptone Water [Bio-Rad] plus 1g/liter sodium pyruvate [Sigma-Aldrich, Aldrich, Fluka, D3435, St Louis, MO, USA], Brila (Merck) which is a brilliant green bile lactose broth, and SEB (Stec Enrichment Broth; Bio-Rad).

After exposure to one of the stress conditions, all strains were individually diluted to a concentration of approximately  $10^2$  cfu of viable cells/ml in the five enrichment media. Similarly, the growth of the eight non-stressed strains was also evaluated. The media were incubated at 37°C and plated in triplicate onto TSA either manually or using the spiral plater after 0, 1, 2, 3, 5, 7 and 24 hours of incubation. All TSA plates were incubated for 24h at 37°C and the colonies were counted. The growth experiments of each individual strain were repeated three times on different occasions.

#### **2.5. *Determination of the growth parameters.***

All counts were log-transformed prior to analyses in the software R (<http://www.R-project.org>). The package 'grofit' was used to fit these data into growth curves using four non-linear growth models, namely the logistic, Gompertz, modified Gompertz and Richards growth models. The "Akaike information criterion" in the software determined the most appropriate model according to the available data. The duration of the lag phase and the maximum growth rate of the resulting models were used for further evaluation.

#### **2.6. *Statistical analyses***

The statistical analyses were performed in STATA/MP 12.1 (Stata Corporation, College Station, TX, USA). The effects of serogroup, stress, and medium on the counts and growth characteristics (lag phase and maximum growth rate) were examined using generalised least squares regressions, including strain as random effect. The significance level of all analyses was set at 0.05.

### 3. Results

#### 3.1. Behavior of non-O157 STEC subjected to stress conditions

The effect of plating non-stressed STEC cells on a selective isolation medium was investigated. No significant difference was observed between the counts of all strains on non-selective TSA and selective TBX media ( $P > 0.05$ ) (data not shown). The selective components in TBX did not inhibit the growth of non-stressed STEC cells.

Table II-2 shows that cold and freeze stress caused lethal injuries in all strains tested, though strain variations were observed. Furthermore, the percentage of lethally injured cells was on average higher after freeze stress compared to cold stress. In contrast to cold and freezing, acid stress did not cause lethal injury for the majority of strains.

Considering the percentage of sub-lethally (stressed) cells, acid stress generally had the least influence, whereas freeze stress caused on average the most sub-lethally injured cells ( $89.1 \pm 8.8\%$ ) (Table II-2).

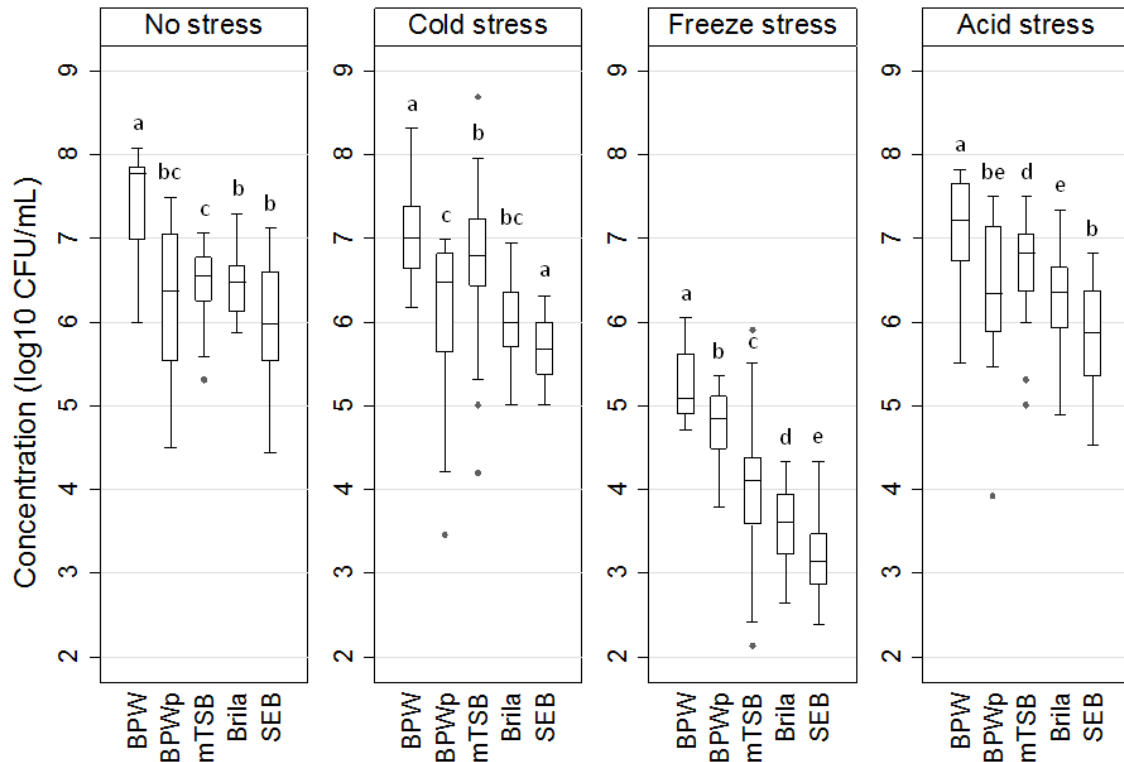
Table II-2. Mean value of the percentage lethal and sub-lethal injury  $\pm$  standard error of the mean of the different STEC strains subjected three times to one of the different stress types. ~ negative percentage (mathematical concept).

stress	% Lethal injury							
	O26 STEC		O103 STEC		O111 STEC		O145 STEC	
	MB 5948	MB 5316	MB 5307	MB 5308	MB 5949	MB 5310	MB 5305	MB 5850
Acid	21.2 $\pm$ 2.9	~	~	~	~	~	~	~
Cold	95.9 $\pm$ 0.3	20.59 $\pm$ 5.8	19.0 $\pm$ 10.0	40.4 $\pm$ 7.4	54.9 $\pm$ 11.0	62.9 $\pm$ 3.8	10.9 $\pm$ 14.3	13.1 $\pm$ 13.6
Freeze	92.5 $\pm$ 0.5	95.7 $\pm$ 1.9	96.7 $\pm$ 1.2	98.4 $\pm$ 0.5	90.6 $\pm$ 2.1	96.3 $\pm$ 1.3	96.2 $\pm$ 1.8	95.6 $\pm$ 0.9
	% Sub-lethal injury							
	O26 STEC		O103 STEC		O111 STEC		O145 STEC	
	MB 5948	MB 5316	MB 5307	MB 5308	MB 5949	MB 5310	MB 5305	MB 5850
Acid	1.3 $\pm$ 1.8	3.5 $\pm$ 9.1	11.2 $\pm$ 8.4	0.7 $\pm$ 4.2	14.9 $\pm$ 4.4	~	~	~
Cold	53.2 $\pm$ 6.6	22.4 $\pm$ 2.9	20.0 $\pm$ 4.2	2.7 $\pm$ 0.7	4.4 $\pm$ 6.0	14.3 $\pm$ 5.5	24.8 $\pm$ 2.4	22.4 $\pm$ 4.4
Freeze	93.4 $\pm$ 0.6	83.0 $\pm$ 1.5	96.5 $\pm$ 1.6	96.5 $\pm$ 2.7	93.9 $\pm$ 0.9	96.2 $\pm$ 2.1	76.2 $\pm$ 2.7	77.5 $\pm$ 2.0

#### 3.2. Growth in different enrichment media

After an enrichment period of seven hours, the highest counts were observed in BPW, regardless of the type of stress applied ( $P < 0.05$ ) (Figure II-1). All stressed strains grew in this seven hours period above 3 log cfu/ml, except for a few freeze stressed STEC strains grown on mTSB, Brila and SEB. After 24 hours of enrichment, regardless of the enrichment medium, strain, or type of stress, all counts varied between 8.26 and 9.11 log cfu/ml.

Figure II-1. Box plot of the concentration, expressed in log colony forming units (CFU) per milliliter, of all STEC strains after seven hours of enrichment in the five enrichment media, subdivided by type of stress applied. The line within the box marks the median. The boundaries of the box represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles. The upper and lower adjacent values are calculated as defined by Tukey (1977) (Tukey, 1977). BPW: Buffered Peptone Water; BPWp: Buffered Peptone Water plus sodium pyruvate; mTSB: Buffered Modified Tryptone Soy Broth; SEB: Stec Enrichment Broth. Significant differences ( $P < 0.05$ ) are indicated with a different alphabetic letter.



Enrichment in BPW and BPWp showed no significant differences in duration of the *lag phase* for non-stressed, acid-stressed, and freeze-stressed cells ( $P > 0.05$ ) (Figure II-2). However, resuscitation of cold-stressed cells in BPWp significantly prolonged the lag phase ( $P < 0.01$ ) compared to BPW. A significantly shorter lag phase was demonstrated after freeze stress in BPW and BPWp compared to the enrichment media mTSB, Brila and SEB ( $P < 0.001$ ). A significantly lower *maximum growth rate* was observed during enrichment on BPWp after cold ( $P < 0.05$ ) and freeze stress ( $P < 0.05$ ) than on BPW (Figure II-3). After acid stress the SEB enrichment showed a significant longer lag phase than all other media ( $P < 0.05$ ) and a significantly lower maximum growth rate as compared to BPW and BPWp ( $P < 0.05$ ). Moreover, the maximum growth rate was significantly lower on SEB during enrichment of non-stressed cells compared to BPW and Brila ( $P < 0.05$ ).

Figure II-2. Box plot of the duration of the lag phase ( $\lambda$ ), expressed in hours, of all STEC strains during the enrichment in the five enrichment media, subdivided by type of stress applied. BPW: Buffered Peptone Water; BPWp: Buffered Peptone Water plus sodium pyruvate; mTSB: Modified Tryptone Soy Broth; SEB: Stec Enrichment Broth. Significant differences ( $P < 0.05$ ) are indicated with a different alphabetic letter.

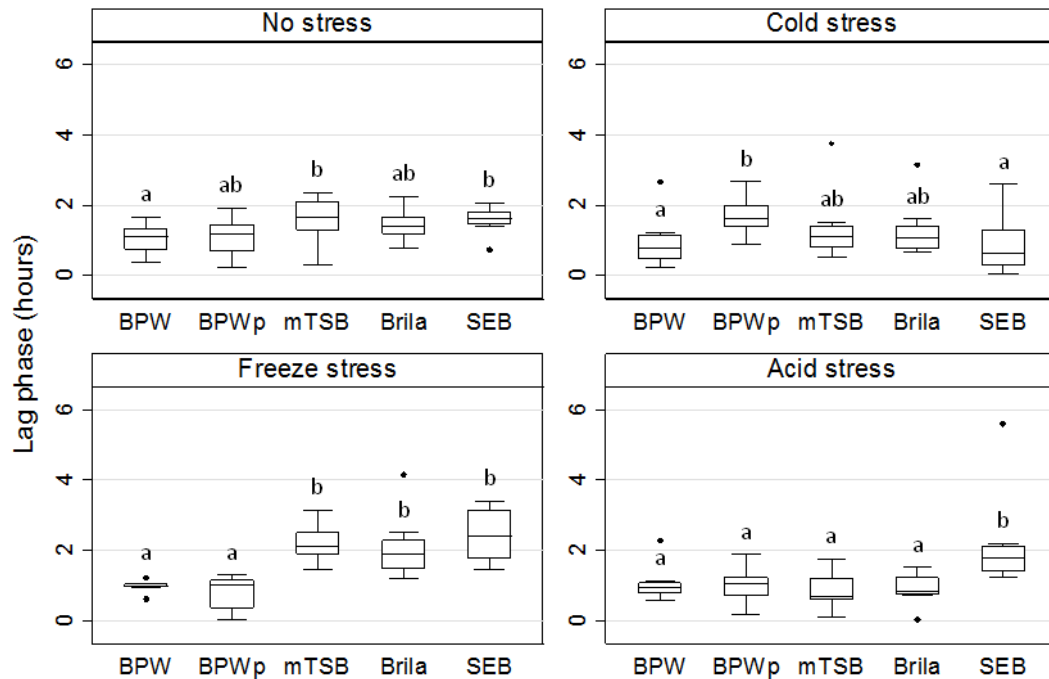
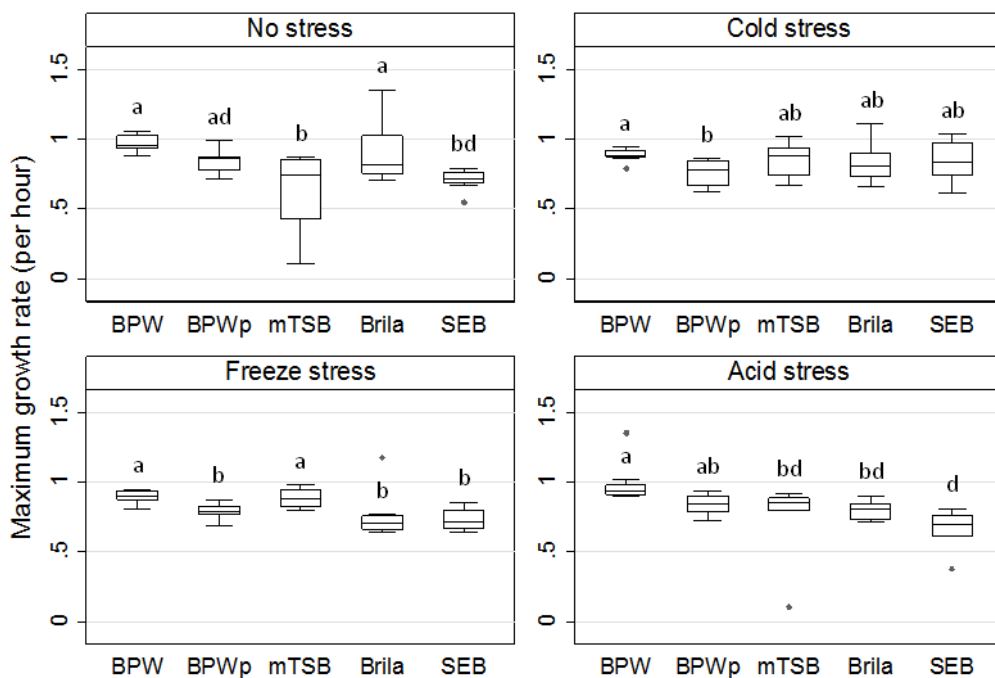


Figure II-3. Box plot of the duration of maximum growth rate ( $\mu_m$ ), expressed in  $\text{hours}^{-1}$ , of all STEC strains during the enrichment in the five enrichment media, subdivided by type of stress. BPW: Buffered Peptone Water; BPWp: Buffered Peptone Water plus sodium pyruvate; mTSB: Buffered Modified Tryptone Soy Broth; SEB: Stec Enrichment Broth. Significant differences ( $P < 0.05$ ) are indicated with a different alphabetic letter.



#### 4. Discussion

The effect of stress on the growth of *E. coli* O157:H7 is well documented; however it cannot be assumed that non-O157 STEC strains will behave similarly to *E. coli* O157:H7 when exposed to the same stress conditions. The acid tolerance observed in the present study has already been described in multiple studies on *E. coli* O157:H7 and non-O157 STEC serogroups (Smith and Fratamico, 2012; M Uyttendaele *et al.*, 2001). To take advantage of this feature, an acid treatment procedure to inhibit the growth of competitor organisms can be used as a preliminary selective step prior to the isolation. Multiple studies have shown the usefulness of this procedure (Fedio *et al.*, 2012; Grant *et al.*, 2009; Tillman *et al.*, 2012). The observed acid tolerance in the present study was comparable to the study of Jasson *et al.* (2007) using the same stress conditions, but their work focused on *E. coli* O157:H7 strains. In our study some strain variations were observed within the STEC serogroups. These findings support the statement of Benjamin and Datta (1995) that the ability of STEC to survive in an acidic environment depends on the strain and not on a specific serogroup. In the present study acid stress was applied during 24 hours at 4°C, thus the resistance mechanism might partly be based on the production of cold shock proteins (Duffy *et al.*, 2006; Smith and Fratamico, 2012). Furthermore, the study was limited to broths acidified with the inorganic acid HCl, but other organic acids, such as acetic acid and citric acid, have inhibited bacterial growth (Molina *et al.*, 2005). In accordance with the results on *E. coli* O157:H7 of Jasson *et al.* (2007), freeze stress induced approximately 90% sub-lethal injury in the small percentage (1.6 to 9.4%) of surviving non-O157 STEC cells. Nevertheless, large outbreaks of STEC infections have been caused by food products that had been frozen prior to consumption. This might imply that these pathogens do not all succumb to the freeze stress they were exposed to. A sufficient number of cells remain viable enough to pose a health threat (Archer, 2004; Dykes, 2006). Therefore, resuscitation in a suitable enrichment medium prior to isolation is essential to avoid false negative test results (Jasson *et al.*, 2007). Consequently, the resuscitation capability of the different tested enrichment media was evaluated during enrichment of freeze stressed cells. The shortest lag phase was observed in BPW and BPWp, which appear to be the most appropriate media to resuscitate this type of stressed cells. Neither medium contains selective agents, thus they are both able to allow resuscitation of injured cells. Griandomenico *et al.* (1997) found that supplementing BPW with sodium pyruvate should ensure better growth and assist in the resuscitation of stressed cells through its protective effect against hydrogen peroxide, which causes damage to cell and DNA in stressed cells. This could not be confirmed in our study, however. Independent of the stress type or absence of stress the duration of the lag phase and maximum growth rate in BPWp were either comparable or even significantly less efficient compared to the regular BPW. Especially for freeze stressed cells, the highest maximum growth

rate was observed in BPW. For both freeze stress and cold stress the lag phase in BPW was also low compared to the other enrichment media. These observations support the ISO/TS 13136:2012, which recommends the use of BPW for the enrichment of stressed bacteria. To ensure a rapid detection of STEC, the growth after seven hours of enrichment was evaluated. Most stressed STEC strains grew in this abbreviated period above the detection limit ( $10^2 - 10^3$  cfu/ml) for real-time PCR screening (Malorny *et al.*, 2003). However, PCR-inhibition and lack of homogeneous pre-PCR samples often influences this theoretical detection limit. Still, some authors state that shorter enrichment periods are more effective to detect positive samples, while others report to the contrary (Hara-Kudo *et al.*, 2000b; Himathongkham *et al.*, 2007; Tutenel, 2003; Vimont *et al.*, 2006a).

Previous studies to compare enrichment media mostly monitored the bacterial growth using absorbance or optical density (OD) measurements (Baylis, 2008; Jasson *et al.*, 2009; Kanki *et al.*, 2011). Although these OD techniques are rapid and relatively easy to automate, they do have important drawbacks, i.e., the relatively high detection limit and underestimation of the lag phase. In order to quantify the growth when the initial inoculum size is below this limit, initial cell counts must be used. The lag phase is then estimated at the intersection between a straight line extrapolated from this count and the slope defined by the maximum growth rate. Besides the technique applied to monitor the bacterial growth, the choice of model used to fit the growth data is of crucial importance (Swinnen *et al.*, 2004). In the present study the growth parameters were determined using a non-linear model.

Eight strains belonging to the most important non-O157 STEC serogroups were used. These strains showed significant variation in the maximum growth rate and duration of the lag phase. In general, growth in BPW resulted in short lag phases followed by high maximum growth rates during the enrichment of pure cultures of all tested strains using all three stress types, resulting in the highest counts after seven hours of enrichment. As mentioned above, supplementing BPW with sodium pyruvate (BPWp) did not result in significantly improved growth parameters compared to BPW. Nonetheless, with or without the addition of pyruvate, BPW might be insufficient for the recovery of a low number of STEC cells in the presence of a high number of competitor organisms. In these cases media containing selective agents might be more effective (Kanki *et al.*, 2011). However, Baylis (2008) remarked that some of these selective enrichment media, even those developed for the isolation of STEC O157, do not support the growth of all STEC strains. For this reason, recently the selectivity of enrichment media for STEC has been modified in various ways. For the most part a variety of concentrations of antibiotics are added to the media to facilitate the growth of STEC by inhibiting the competitor organisms. Nevertheless, some STEC strains show sensitivity to these antibiotics, which remains a significant

drawback. Moreover, it has been proven that cells might become antibiotic-sensitive while in a state of stress, which may turn these antibiotics into inhibitors during the resuscitation phase (Hara-Kudo *et al.*, 2000b; Kanki *et al.*, 2009). For this reason antibiotics are often added after a resuscitation phase or other selective agents are used such as bile salts or brilliant green. Tzschoppe *et al.* (2012) observed approximately a 50% reduction in competitor flora without decreasing the numbers of detectable STEC cells when using Brila instead of BPW for the detection and isolation of STEC from ready-to-eat vegetables. In the present study, Brila and the novel SEB media were the most selective enrichment media of the five examined. Still, the selective agents present in these media were sufficient to effectively hamper the resuscitation and growth of the stressed pure STEC cultures compared to BPW. In general, lag phase and maximum growth rate using Brila were shorter and higher, respectively, compared to the use of SEB. This resulted in higher counts on Brila than on SEB after seven hours of enrichment.

In conclusion, the present study clearly shows that the low-nutrient non-selective enrichment medium BPW is the best option to resuscitate very or slightly stressed STEC cells. When applying a abbreviated enrichment period of seven hours, BPW resulted in the highest growth. Nevertheless, its performance in a more complex environment with different physico-chemical properties and interfering microflora still needs further research. For more selective media, Brila performs better compared to SEB. In further research of our group, the enrichment capabilities of Brila using a more complex environment will be investigated and compared with BPW.

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## **Chapter 3: Comparison of six chromogenic agar media for the isolation of a broad variety of non-O157 Shiga toxin-Producing *Escherichia coli* (STEC) serogroups**

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Adapted from:

Verhaegen B., De Reu K., Heyndrickx M., De Zutter L. (2015) Comparison of Six Chromogenic Agar Media for the Isolation of a Broad Variety of Non-O157 Shigatoxin-Producing *Escherichia coli* (STEC) Serogroups. International Journal of Environmental Research and Public Health., 12, 6965-6978.

**Abstract**

The isolation of non-O157 STEC from food samples has proved to be challenging. The selection of a suitable selective isolation agar remains problematic. The purpose of this study was to qualitatively and quantitatively evaluate six chromogenic agar media for the isolation of STEC: Tryptone Bile X-glucuronide agar (TBX), Rainbow® Agar O157 (RB), Rapid *E. coli* O157:H7 (RE), Modified MacConkey Agar (mMac), CHROMagar™ STEC (Chr ST) and chromID™ EHEC (Chr ID). During this study, 45 *E. coli* strains were used, including 39 STEC strains belonging to 16 different O serogroups and 6 non-STEC *E. coli*. All *E. coli* strains were able to grow on TBX and RB, whereas one STEC strain was unable to grow on Chr ID and a number of other STEC strains did not grow on mMac, CHROMagar STEC and Rapid *E. coli* O157:H7. However, only the latter three agars were selective enough to completely inhibit the growth of the non-STEC *E. coli*. Our conclusion was that paired use of a more selective agar such as CHROMagar STEC together with a less selective agar like TBX or Chr ID might be the best solution for isolating non-O157 STEC from food.

## 1. Introduction

The multitude of infectious diseases transmitted by micro-organisms is a burden for public health. The well-known Shigatoxin-producing *Escherichia coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC), causes human infection through direct transmission from person to person or from infected animals. It can also be indirectly transmitted via contaminated food, water, or environments contaminated with faeces (EFSA, 2014). STEC infections can be responsible for clinical symptoms ranging from mild to severe diarrhea, possibly complicated with hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Piérard *et al.*, 2012). Rapid detection of this pathogen is of utmost importance to ensure appropriate action to safeguard public health. The recently increased use of highly-automated real-time PCR screening techniques provides the required highly-sensitive detection of all STEC. However, the follow-up culture-based isolation of the pathogen can be labour-intensive and time-consuming due to the long incubation period. In some cases such isolation is even unsuccessful due to lack of sufficiently selective isolation media (Franz *et al.*, 2014). Because, STEC O157:H7 was initially the most common serotype within the STEC group, the development of isolation media has been targeted for this serotype. The current cultural method of STEC O157:H7 is based on its inability to ferment sorbitol, its lack of  $\beta$ -D-glucuronidase enzyme activity and its resistance to selective agents such as potassium tellurite, novobiocin and cefixime (Mathusa *et al.*, 2010). Consequently, multiple selective isolation media with chromogenic substrates have been formulated for the isolation of *E. coli* O157:H7 (Perry and Freydière, 2007). These isolation media fail to detect atypical O157 STEC in addition to a large number of non-O157 STEC strains. However, these strains are increasingly recognised and reported as important foodborne pathogens worldwide; an important example is the STEC O104:H4 outbreak in Germany and France of 2011. In Europe the most frequently isolated and human pathogenic most important non-O157 STEC serogroups are O26, O91, O103, O111 and O145 (EFSA, 2014; Mainil and Daube, 2005; Wang *et al.*, 2013). In contrast to *E. coli* O157:H7, strains of these serogroups exhibit a broad variety of biochemical characteristics and a different sensitivity to selective agents. No single chromogenic isolation medium has yet been developed that allows cultivation and differentiation of all STEC from food samples (Kalchayanand *et al.*, 2013). Nevertheless, obtaining a verified positive isolate is crucial to confirm the positive results of the PCR-based screening techniques. In addition, culturing makes it possible to type the isolate in order to establish possible contamination routes and reveal important virulence factors (Kase *et al.*, 2015).

Currently, the International Organization for Standardization (ISO) states that all samples in which a *stx* gene has been detected by PCR after enrichment should be further investigated by

an isolation step on a selective agar medium. The use of Tryptone Bile X-glucuronide agar (TBX) for isolation of STEC is recommended. Since this medium lacks selectivity for STEC multiple presumptive positive colonies (up to 50) are routinely confirmed, and the choice of another medium is therefore allowed (ISO, 2012a). For this purpose a number of agar media has been developed; they can be either specific for isolation of *E. coli* O157, O26, or for all STEC in general. The growth capabilities and morphologies of many STEC serotypes on these selective media have not been thoroughly investigated, however.

The aim of this study was to evaluate the growth capacity and colony colours of a broad variety of STEC serotypes on several chromogenic media used for the isolation of *E. coli* O157 and other STEC, regardless of the O serogroup. The features of some non-STEC *E. coli* were also investigated on those media as a comparison (Quiros *et al.*, 2015).

## **2. Material and methods**

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### **2.1. Strains**

Table III-1 lists the 45 *E. coli* strains used in this study. A total of 39 STEC belonging to the four most common and 12 less common non-O157 STEC serogroups were examined. Most strains were isolated from human patients by the Belgian National Reference Centre (UZ Brussels, Belgium, Prof. D. Piérard); others originated from food samples in Belgium. In addition, six non-STEC *E. coli* were included: two enteropathogenic *E. coli* (EPEC) and four commensal *E. coli* all isolated from cattle faeces. All strains were stored at -80°C using Pro-Lab Microbank cryovials (Pro-Lab, Ontario, Canada) according to the manufacturer's instructions. Presence of *stx1*, *stx2*, *eae* and *hlyA* genes was analysed according to Botteldoorn *et al.* (2003) (Botteldoorn *et al.*, 2003). For the *ter B* gene presence the method described by Taylor *et al.* (2002) (Taylor *et al.*, 2002) was used. The strains were cultured onto Tryptone Soy Agar (TSA; Oxoid, Ltd., Basingstroke, Hampshire, England) at 37°C for 24h. A single colony from these culture plates was transferred into Tryptone Soy Broth (TSB; Oxoid) and incubated at 37°C for 24h.

Table III-1. Overview of STEC, EPEC and commensal *E. coli* strains.

Strains	Serotypes	Origin	Virulence genes				
			<i>stx 1</i>	<i>stx 2</i>	<i>eae</i>	EHEC- <i>hlyA</i>	<i>terB</i>
MB 5323	O5:H-	human	+	-	-	+	-
MB 5324	O5:H-	human	+	-	+	+	+
MB 5321	O8:H-	human	-	+	-	-	-
MB 5322	O8:H9	human	-	+	-	-	-
MB 5325	O55:H12	human	+	-	-	-	+
MB 5312	O55:H7	human	-	+	+	-	-
MB 5342	O63:H6	human	-	+	+	+	-
MB 5313	O63:H6	human	-	+	+	-	-
MB 5334	O84:H-	human	-	+	+	+	+
MB 5333	O84:H28	human	+	-	+	+	+
MB 5336	O91:H-	human	-	+	-	+	-
MB 5335	O91:H21	human	-	+	-	+	-
MB 5339	O113:H2	human	-	+	+	-	-
MB 5338	O113:H21	human	-	+	-	-	+
MB 5950	O118:H16	human	+	-	+	+	+
MB 5951	O118:H16	human	+	-	+	-	+
MB 5337	O121:H19	human	-	+	+	+	+
MB 5326	O128:H-	human	+	+	-	+	-
MB 5327	O128:H-	human	-	+	+	-	-
MB 5329	O146:H28	human	-	+	-	-	+
MB 5328	O146:H-	human	+	+	-	+	+
MB 5340	O182:H34	human	+	-	+	+	+
MB 5341	O182:H25	human	+	-	+	+	+
MB 5948	O26:H11	swab*	-	+	+	+	+
MB 5316	O26:H11	milk*	+	-	+	+	+
MB 2658	O26:H11	human	+	-	+	+	+
MB 2775	O26:H11	human	-	+	+	+	+
MB 5307	O103:H2	swab	+	-	+	+	-
MB 5308	O103:H2	milk	+	-	+	+	-
MB 2654	O103:H2	human	+	-	+	+	-
MB 2651	O103:H2	human	+	-	+	+	-
MB 5949	O111:H2	swab	-	+	+	+	+
MB 5310	O111:H8	swab	+	+	+	+	+
MB 2679	O111:H-	human	+	-	+	+	+
MB 2685	O111:H-	human	+	+	+	+	+
MB 5305	O145:H28	swab	+	-	+	+	+
MB 5850	O145:H28	swab	-	+	+	+	+
MB 2655	O145:H-	human	+	-	+	+	+
MB 2820	O145:H-	human	+	+	+	+	+
MB 5952		cattle faeces	-	-	+		+
MB 5953		cattle faeces	-	-	+		+
MB 5956		cattle faeces	-	-	-		-
MB 5957		cattle faeces	-	-	-		-
MB 5958		cattle faeces	-	-	-		-
MB 5959		cattle faeces	-	-	-		-

\*Swab: cattle carcass swab; milk: cow milk

## **2.2. Selective isolation media.**

The following selective isolation media were evaluated. Tryptone Bile X-glucuronide agar (TBX; Bio-Rad, Marnes-la-Coquettes, France); Rainbow® Agar O157 (RBA; Biolog Inc., Hayward, CA, USA) without supplementations; Rapid *E. coli* O157:H7 (RE; Bio-Rad), supplemented with 10 mg/l novobiocin and 0.8 mg/l potassium tellurite; Modified MacConkey Agar (mMac) for the isolation of non-O157 STEC strains as described by Possé *et al.* (2008). Briefly, this medium contains MacConkey agar base (BD Biosciences, Franklin Lakes, NJ) supplemented with two sugars (sucrose and sorbose) and several selective components: 3.5 g/l bile salts No. 3 (Sigma Aldrich, Fluka, St-Louis, USA), 0.05 g/l 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-gal, Glycosynth, Warrington, UK), 0.05 g/l isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG, Glycosynth), 8.0 mg/l novobiocin (Sigma) and 2.5 mg/l potassium tellurite (Sigma); CHROMagar™ STEC supplemented with 10 ml/l selective mix (Chr ST; CHROMagar Microbiology, Paris, France) and the recently launched ChromID™ EHEC supplemented with 4 ml/l cefixime-tellurite mix (Chr ID; bioMérieux, Paris, France) were included.

## **2.3. Qualitative study**

After incubation 10  $\mu$ l of each TSB strain culture was inoculated onto the six chromogenic agar media. All agar media were incubated at 37°C for 24h and visually examined for growth and colony morphology.

## **2.4. Quantitative study**

To determine the possible inhibition of growth of the STEC strains on the chromogenic agar media, all cultures grown in TSB were serially diluted in Peptone Water (Bio-Rad) to a concentration of  $10^4$  cfu/ml. One millilitre of each dilution was manually spread plated on two agar plates (each 0.5 ml) of each of the six agar media and TSA medium as reference. In addition 100  $\mu$ l of each dilution was inoculated on one agar plate of each of the seven agar media using a spiral plate machine (Eddy Jet Spiral Plater, IUL instruments, Barcelona, Spain). The plates were incubated for 24h at 37°C and the colonies counted. In accordance with Gill *et al.* (2014) the efficiency of recovery was calculated as the percentage of counted colonies on the different selective isolation media compared to the enumeration obtained on the non-selective TSA. The study was replicated three-fold; the mean recovery percentage and standard error were calculated.

### **3. Results and Discussion**

The isolation of non-O157 STEC strains from food samples has proved challenging due to the lack of known differential biochemically characteristics and inherent sensitivities to additives. For this reason the discrimination from other *E. coli* and other non-target organisms remains problematic. (Kalchayanand *et al.*, 2013; Possé *et al.*, 2008). Before the interference of an extensive background microbiota can be evaluated, the growth capabilities of STEC strains themselves should be investigated. In this study the growth of STEC on six chromogenic agar media was evaluated using a range of STEC serotypes often isolated in Belgium. Furthermore, the growth and appearance of these STEC strains was compared to some common non-STEC *E. coli*. In the threefold replicated experiment some natural variation in counts was observed, but the colony colours of the different cultures of STEC strains, based upon the enzymatic cleaving of chromogenic substrates and carbohydrate fermentation, remained the same.

#### **3.1. Tryptone Bile X-glucuronide agar (TBX).**

Among the six evaluated chromogenic media, TBX is designed to detect all *E. coli*, including STEC. Therefore, all tested STEC and non-STEC *E. coli* strains were able to grow on TBX (Table III-2) and demonstrated the typical blue-green colour indicating the presence of  $\beta$ -glucuronidase activity (Figure III-1 a). Moreover, the recovery percentage of the STEC serogroups and other non-STEC *E. coli* compared to TSA was at least 78% and 93%, respectively (Table III-3), with averages for both groups of 101% and 97%, respectively. This ISO/TS 13136:2012 recommended agar medium contains selective agents inhibiting the growth of Gram-positive organisms and swarming by *Proteus* sp. (Evans *et al.*, 2008). It remains a valuable isolation medium for STEC, despite the labour-intensity and time-consuming practice of isolating multiple presumptive positive colonies (up to 50), pooling and confirming as described in the ISO/TS, due to its low selectivity.

Table III-2. Growth and colony appearance of STEC and non-STEC *E. coli* strains on six chromogenic isolation media. TBX: Tryptone Bile X-glucuronide agar, RBA: Rainbow® Agar O157, RE: Rapid *E. coli* O157:H7, mMac: Modified MacConkey Agar as described by Possé *et al.* (2008), Chr ST: CHROMagar STEC™, Chr ID: Chrom ID EHEC.

strain	Serotype	<i>TerB</i>	TBX	RBA	RE	mMac	Chr ST	Chr ID
MB 5323	O5:H-	-	blue-green	grey-green	green	-*	-	purple
MB 5324	O5:H-	+	blue-green	grey-green	green	green	mauve	purple
MB 5321	O8:H-	-	blue-green	red	green	-	-	purple
MB 5322	O8:H9	-	blue-green	purple	green	-	-	-
MB 5325	O55:H12	+	blue-green	purple	green	grey-green	mauve	blue
MB 5312	O55:H7	-	blue-green	red	green	-	-	purple
MB 5342	O63:H6	-	blue-green	red	dark blue	-	-	purple
MB 5313	O63:H6	-	blue-green	red	dark blue	-	-	purple
MB 5334	O84:H-	+	blue-green	cream-white	yellow	yellow	mauve	white
MB 5333	O84:H28	+	blue-green	red-purple	green	green	mauve	purple
MB 5336	O91:H-	-	blue-green	purple	green	-	-	purple
MB 5335	O91:H21	-	blue-green	purple	green	-	-	purple
MB 5339	O113:H2	-	blue-green	red-purple	green	-	-	purple
MB 5338	O113:H21	+	blue-green	red-purple	green	grey-green	mauve	purple
MB 5950	O118:H16	+	blue-green	purple	green	red	mauve	purple
MB 5951	O118:H16	+	blue-green	purple	green	grey-green	mauve	purple
MB 5337	O121:H19	+	blue-green	red	yellow	red	mauve	red
MB 5326	O128:H-	-	blue-green	red-purple	green	-	-	purple
MB 5327	O128:H-	-	blue-green	red-purple	green	-	-	purple
MB 5329	O146:H28	+	blue-green	purple	green	green	mauve	purple
MB 5328	O146:H-	+	blue-green	purple	green	grey-green	mauve	purple
MB 5340	O182:H34	+	blue-green	purple	dark blue	green	mauve	purple
MB 5341	O182:H25	+	blue-green	purple	dark blue	green	mauve	purple
MB 5948	O26:H11	+	blue-green	purple	green	red	mauve	purple
MB 5316	O26:H11	+	blue-green	purple	green	red	mauve	purple
MB 2658	O26:H11	+	blue-green	purple	green	red	mauve	purple
MB 2775	O26:H11	+	blue-green	purple	green	red	mauve	green
MB 5307	O103:H2	-	blue-green	purple	green	blue-green	mauve	purple
MB 5308	O103:H2	-	blue-green	purple	green	blue-green	mauve	purple
MB 2654	O103:H2	-	blue-green	purple	green	-	-	purple
MB 2651	O103:H2	-	blue-green	purple	green	-	-	purple
MB 5949	O111:H2	+	blue-green	grey-green	green	grey-green	mauve	purple
MB 5310	O111:H8	+	blue-green	grey-green	green	grey-green	mauve	purple
MB 2679	O111:H-	+	blue-green	grey-green	green	grey-green	mauve	purple
MB 2685	O111:H-	+	blue-green	grey-green	green	grey-green	mauve	purple
MB 5305	O145:H28	+	blue-green	purple	green	green	mauve	purple
MB 5850	O145:H28	+	blue-green	purple	green	green	mauve	purple
MB 2655	O145:H-	+	blue-green	purple	green	green	mauve	purple
MB 2820	O145:H-	+	blue-green	purple	green	green	mauve	purple
MB 5952		+	blue-green	purple	green	green	mauve	purple
MB 5953		+	blue-green	purple	green	green	mauve	green
MB 5956		-	blue-green	purple	-	-	-	red
MB 5957		-	blue-green	purple	-	-	-	purple
MB 5958		-	blue-green	purple	-	-	-	purple
MB 5959		-	blue-green	purple	green	-	-	purple

-: no growth.



Table III-3. The mean recovery percentages of the STEC and non-STEC strains  $\pm$  standard error on the six chromogenic isolation media compared with the growth on TSA medium (the average concentration of the inocula based on the logarithmic counts on Tryptone Soy Agar (TSA):  $4.13 \pm 0.12 \log_{10}$  cfu/ml). TBX: Tryptone Bile X-glucuronide agar, RB: Rainbow® Agar O157, RE: Rapid *E. coli* O157:H7, mMac: Modified MacConkey Agar as described by Possé *et al.* (2008), Chr ST: CHROMagar STEC, Chr ID: ChromID EHEC.

Strain	Serotype	<i>TerB</i>	TBX	RB	RE	mMac	Chr ST	Chr ID
MB 5323	O5:H-	-	116 $\pm$ 15	114 $\pm$ 10	-*	-	-	<1
MB 5324	O5:H-	+	91 $\pm$ 10	104 $\pm$ 9	-	60 $\pm$ 9	60 $\pm$ 18	85 $\pm$ 26
MB 5321	O8:H-	-	94 $\pm$ 4	95 $\pm$ 10	-	-	-	41 $\pm$ 13
MB 5322	O8:H9	-	97 $\pm$ 8	109 $\pm$ 15	2 $\pm$ 1	-	-	-
MB 5325	O55:H12	+	84 $\pm$ 8	119 $\pm$ 15	-	51 $\pm$ 18	34 $\pm$ 7	42 $\pm$ 18
MB 5312	O55:H7	-	110 $\pm$ 16	114 $\pm$ 11	-	-	-	30 $\pm$ 4
MB 5342	O63:H6	-	90 $\pm$ 18	68 $\pm$ 10	-	-	-	33 $\pm$ 10
MB 5313	O63:H6	-	92 $\pm$ 8	90 $\pm$ 18	-	-	-	78 $\pm$ 25
MB 5334	O84:H-	+	107 $\pm$ 11	89 $\pm$ 15	-	101 $\pm$ 21	108 $\pm$ 14	107 $\pm$ 36
MB 5333	O84:H28	+	96 $\pm$ 6	106 $\pm$ 9	-	86 $\pm$ 33	64 $\pm$ 7	68 $\pm$ 21
MB 5336	O91:H-	-	82 $\pm$ 15	108 $\pm$ 2	-	-	-	<1
MB 5335	O91:H21	-	95 $\pm$ 32	99 $\pm$ 9	-	-	-	34 $\pm$ 7
MB 5339	O113:H2	-	115 $\pm$ 12	108 $\pm$ 4	30 $\pm$ 8	-	-	26 $\pm$ 7
MB 5338	O113:H21	+	78 $\pm$ 23	108 $\pm$ 5	-	29 $\pm$ 8	51 $\pm$ 12	38 $\pm$ 10
MB 5950	O118:H16	+	95 $\pm$ 5	101 $\pm$ 20	-	36 $\pm$ 6	57 $\pm$ 10	32 $\pm$ 7
MB 5951	O118:H16	+	104 $\pm$ 18	116 $\pm$ 5	-	47 $\pm$ 14	101 $\pm$ 15	54 $\pm$ 8
MB 5337	O121:H19	+	96 $\pm$ 1	93 $\pm$ 5	-	142 $\pm$ 49	122 $\pm$ 12	72 $\pm$ 9
MB 5326	O128:H-	-	88 $\pm$ 12	36 $\pm$ 9	-	-	-	26 $\pm$ 6
MB 5327	O128:H-	-	84 $\pm$ 31	81 $\pm$ 11	-	-	-	<1
MB 5329	O146:H28	+	94 $\pm$ 15	93 $\pm$ 10	-	6 $\pm$ 6	33 $\pm$ 9	33 $\pm$ 9
MB 5328	O146:H-	+	125 $\pm$ 20	94 $\pm$ 7	-	26 $\pm$ 1	6 $\pm$ 1	26 $\pm$ 4
MB 5340	O182:H34	+	110 $\pm$ 12	88 $\pm$ 4	-	44 $\pm$ 2	33 $\pm$ 4	85 $\pm$ 19
MB 5341	O182:H25	+	100 $\pm$ 3	116 $\pm$ 12	-	33 $\pm$ 8	30 $\pm$ 10	18 $\pm$ 3
MB 5948	O26:H11	+	83 $\pm$ 15	107 $\pm$ 2	-	<1	60 $\pm$ 19	<1
MB 5316	O26:H11	+	89 $\pm$ 20	88 $\pm$ 6	-	100 $\pm$ 24	82 $\pm$ 16	15 $\pm$ 5
MB 2658	O26:H11	+	99 $\pm$ 6	96 $\pm$ 17	-	<1	48 $\pm$ 16	6 $\pm$ 0
MB 2775	O26:H11	+	116 $\pm$ 6	88 $\pm$ 11	2 $\pm$ 1	2 $\pm$ 0	35 $\pm$ 6	29 $\pm$ 2
MB 5307	O103:H2	-	113 $\pm$ 9	106 $\pm$ 7	-	-	-	73 $\pm$ 12
MB 5308	O103:H2	-	109 $\pm$ 7	112 $\pm$ 5	-	-	-	63 $\pm$ 7
MB 2654	O103:H2	-	119 $\pm$ 17	105 $\pm$ 11	-	-	-	98 $\pm$ 5
MB 2651	O103:H2	-	93 $\pm$ 0	110 $\pm$ 2	-	-	-	79 $\pm$ 13
MB 5949	O111:H2	+	114 $\pm$ 8	113 $\pm$ 1	-	29 $\pm$ 9	24 $\pm$ 4	50 $\pm$ 6
MB 5310	O111:H8	+	110 $\pm$ 14	98 $\pm$ 5	-	23 $\pm$ 3	47 $\pm$ 7	49 $\pm$ 15
MB 2679	O111:H-	+	122 $\pm$ 8	113 $\pm$ 5	-	43 $\pm$ 6	59 $\pm$ 15	44 $\pm$ 15
MB 2685	O111:H-	+	100 $\pm$ 2	100 $\pm$ 14	-	39 $\pm$ 11	65 $\pm$ 7	71 $\pm$ 4
MB 5305	O145:H28	+	120 $\pm$ 17	121 $\pm$ 16	-	49 $\pm$ 13	40 $\pm$ 10	26 $\pm$ 7
MB 5850	O145:H28	+	96 $\pm$ 10	87 $\pm$ 10	-	42 $\pm$ 10	72 $\pm$ 22	4 $\pm$ 1
MB 2655	O145:H-	+	112 $\pm$ 13	96 $\pm$ 15	-	67 $\pm$ 13	23 $\pm$ 5	25 $\pm$ 6
MB 2820	O145:H-	+	100 $\pm$ 8	102 $\pm$ 23	-	51 $\pm$ 7	24 $\pm$ 5	4 $\pm$ 0
MB 5952		+	96 $\pm$ 13	111 $\pm$ 9	7 $\pm$ 3	56 $\pm$ 11	60 $\pm$ 18	72 $\pm$ 21
MB 5953		+	116 $\pm$ 12	110 $\pm$ 21	17 $\pm$ 9	29 $\pm$ 21	66 $\pm$ 11	50 $\pm$ 10
MB 5956		-	96 $\pm$ 13	102 $\pm$ 19	-	-	-	11 $\pm$ 9
MB 5957		-	116 $\pm$ 12	118 $\pm$ 36	-	-	-	15 $\pm$ 14
MB 5958		-	98 $\pm$ 19	118 $\pm$ 21	-	-	-	4 $\pm$ 2
MB 5959		-	93 $\pm$ 13	72 $\pm$ 17	2 $\pm$ 1	-	-	1 $\pm$ 1

\*-: no growth (<1 cfu/ml)

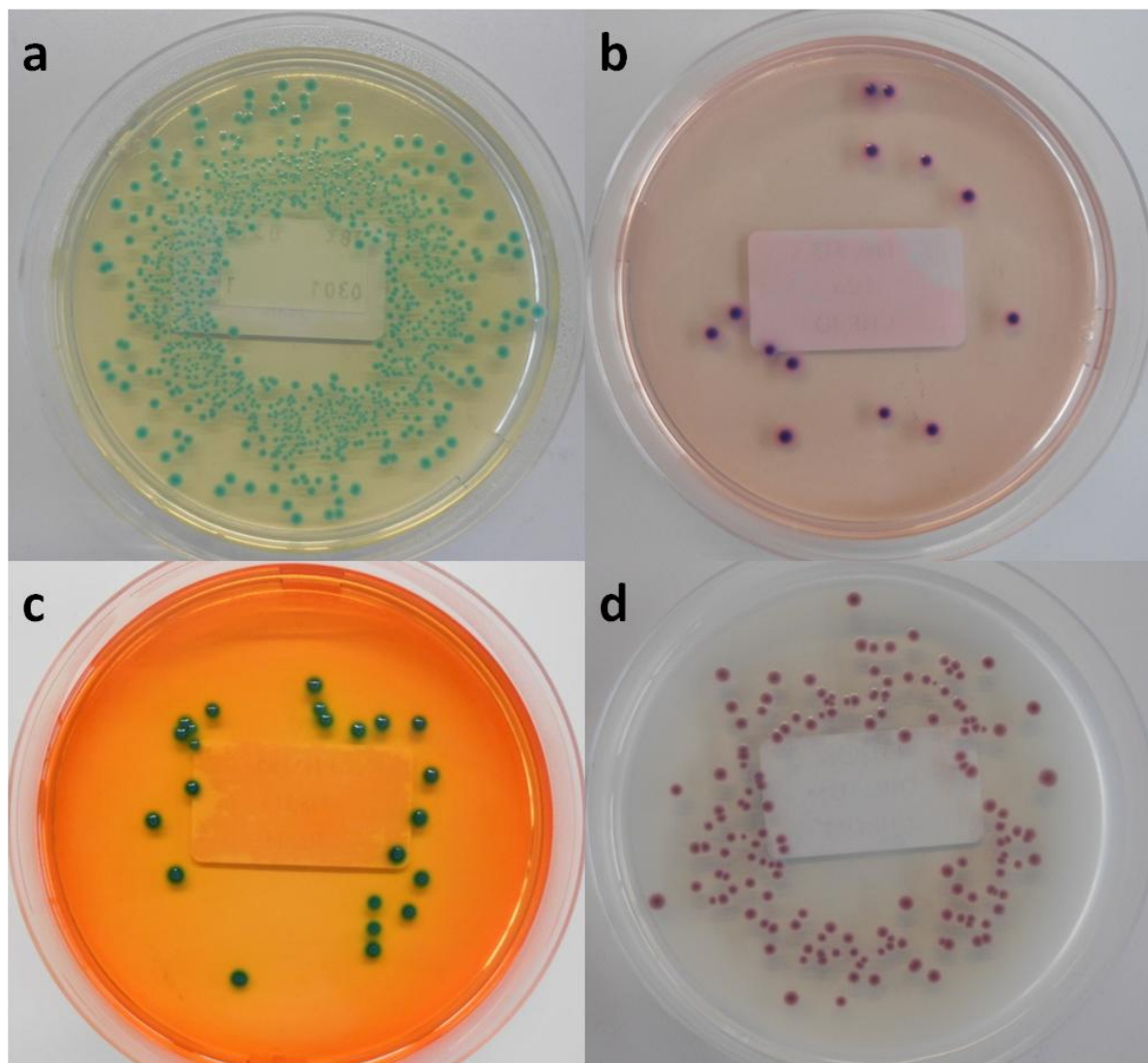


Figure III-1. Macroscopic view of a: Tryptone Bile X-glucuronide agar (TBX), b: chromID EHEC agar (Chr ID), c: Rapid *E. coli* O157:H7 agar (RE) and d: CHROMagar STEC (Chr ST), inoculated with MB 5316 (a), MB 5948 (b), MB 5322 (c) and MB 5341 (d).

### 3.2. *Rainbow® Agar O157(RBA)*

In accordance with TBX, RBA was able to support the growth of all tested STEC and non-STEC *E. coli* strains, and the average recovery percentage of the STEC serogroups and other non-STEC *E. coli* was similar this for TSA (approximately 100%). The colony colours of the different strains on RB ranged from purple – red – pink - grey-green - to cream-white (Figure III-2). Remarkably, the colonies of strains belonging to the same O-serogroup were not always consistent in color. Non-STEC *E. coli* strains could not be differentiated from STEC strains based on colony colours.

Modifications to this RBA base have been described to support growth of STEC while allowing more selectivity towards non-target organisms. Supplementation of the medium with 0.8 mg/l potassium tellurite and 10 mg/l sodium novobiocin is recommended by the manufacturer for samples with high microbiological background, whereas 0.05 mg/l cefixime, 0.15 mg/l potassium

tellurite, and 5 mg/l novobiocin is recommended by the USDA STEC (Tillman *et al.*, 2012). However, multiple studies demonstrated that both supplementations were unable to support the growth of a substantial proportion of STEC strains tested (Gill *et al.*, 2014; Kase *et al.*, 2015; Wheeler *et al.*, 2015). Further, Kase *et al.* (2015) showed that the addition of washed sheep's blood to RBA substantially reduced these inhibitions. However, in the present study only the Rainbow agar base without any supplementations was evaluated. The observed broad variety of colony colours and the inability to distinguish between STEC and non-STEC *E. coli* was considered problematic to select colonies for confirmation testing.

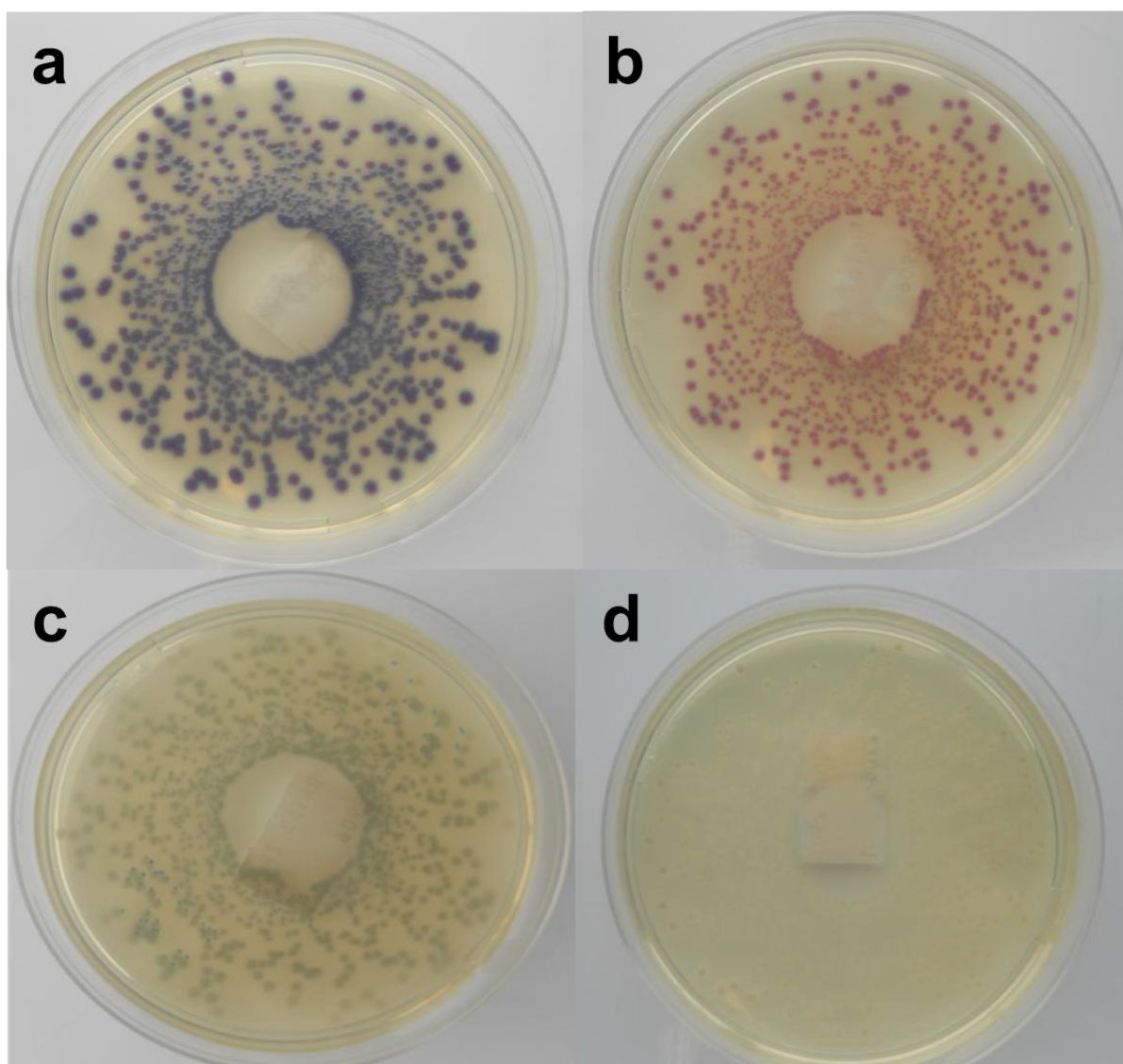


Figure III-2. Macroscopic view of Rainbow O157 agar inoculated with STEC strains, a: purple colonies (MB 5322), b: red colonies (MB 5313), c: grey-green colonies (MB 5324) and d: cream-white colonies (MB 5334).

### 3.3. *Rapid E. coli O157:H7 (RE)*

In the qualitative study RE supported the growth of all tested STEC strains and half of the non-STECC *E. coli* strains. All colonies showed a green morphology (Figure III-1 c), except for two STEC serogroups (O63, O182), which presented a characteristic dark blue colour typical for *E. coli* O157:H7. When RE was inoculated with lower concentrated inocula only three STEC and three non-STECC *E. coli* strains were able to form colonies, with a very low average recovery percentage of 11% and 9%, respectively. In general, RE showed a significant selectivity towards STEC in both colony morphology and growth inhibition, making this agar medium unsuitable for isolation of non-O157 STEC strains.

### 3.4. *CHROMagar™ STEC (Chr ST)*

In the present study only 24 STEC strains (61%) were able to develop colonies after inoculation at the low dose on Chr ST. The STEC and non-STECC *E. coli* strains that were able to grow presented colonies in many shades of mauve and often displayed different edges (Figure III-1 d). Moreover, the average recovery percentage was 53% and 63% compared to TSA, respectively. This high inhibition has already been observed in multiple studies carried out since the launch of Chr ST (Gill *et al.*, 2014; Hirvonen *et al.*, 2012; Kase *et al.*, 2015; Tzschoppe *et al.*, 2012; Wylie *et al.*, 2013). Both Tzschoppe *et al.* (2012) and Hirvonen *et al.* (2012) remarked on strong association between the growth on Chr ST and the presence of *terB* and *terD* of the *ter* gene cluster, respectively. This gene complex contains four essential genes (*terB*, *terC*, *terD* and *terE*) conferring the resistance to strong oxidizing agent tellurite. These tellurite-resistant bacteria reduce tellurite to its less toxic form, which accumulates as black pigment inside the cell (Aradská *et al.*, 2013; Chiang *et al.*, 2008; Orth *et al.*, 2007). In the present study *terB* was selected as marker for the *ter* gene cluster. Twenty-four of the tested STEC strains were *terB*-positive and all were able to grow on Chr ST. Furthermore, all non-STECC *E. coli* strains failed to develop colonies, except the two *terB*-positive EPEC strains. This finding confirms the strong association between the growth on Chr ST and the presence of *terB*.

Moreover, it was observed that only a small proportion of the *ea*-negative STEC strains were able to grow (Gill *et al.*, 2014; Hirvonen *et al.*, 2012; Kase *et al.*, 2015; Tzschoppe *et al.*, 2012). In the present study 10 *ea*-negative strains were included; only four grew on Chr ST.

### 3.5. *Modified MacConkey Agar (mMac)*

mMac was originally designed to differentiate between the four most common non-O157 STEC serogroups (O26, O103, O111, O145), using the colony colours dependent on the  $\beta$ -D-galactosidase activity and carbohydrate fermentation of these four serogroups (Figure III-3). While the tested

STEC strains belonging to this serogroup presented the predicted colony colours, the growth of two O103 STEC strains was not supported. Most of the other serogroups showed similar colours, while some exhibited atypical colours (yellow). In accordance with RBA, the colonies of strains of the same O-serogroup were not always consistent in colour. Moreover, the color differences found in our study were often subtle and hard to discriminate. Still, Verstraete *et al.* (2012) indicated its effectiveness as isolation medium for non-O157 STEC in food during the validation by an international ring trial. Nevertheless, the same STEC and non-STEC *E. coli* strains that failed to develop colonies on Chr ST also failed on mMac. The STEC that were able to grow showed an average recovery percentage of 50% compared to TSA. On the other hand, all non-STEC *E. coli* strains failed to develop colonies, except the two EPEC strains whose morphology was similar to the O145 STEC strains. Similar to Chr ST, a correlation between the growth on mMac and the presence of *terB* was observed.

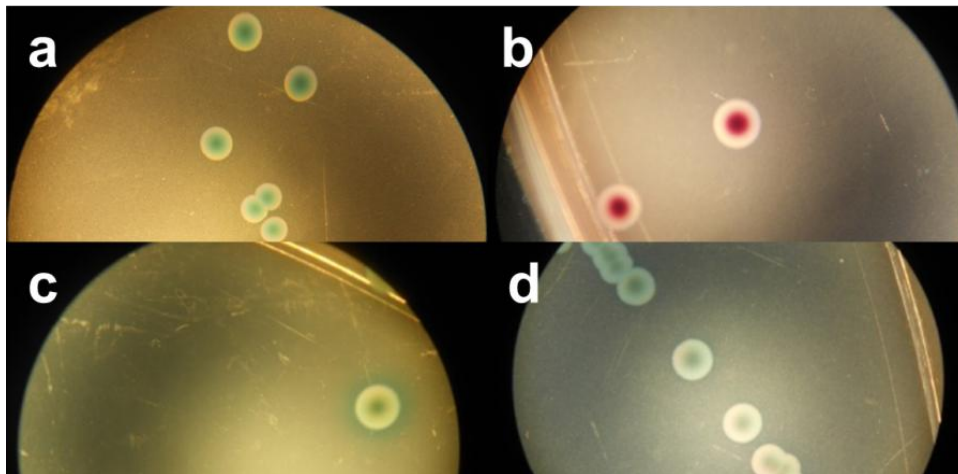


Figure III-3. Stereo-microscopic view (a, b, c, d) of modified MacConkey agar as described by Possé *et al.* (2008), inoculated with STEC strains. (a: STEC O145 (MB 5850) , b: STEC O26 (MB 5316), c: STEC O103 (MB 5307) and d: STEC O111 (MB 2679)).

### 3.6. ChromID EHEC (Chr ID)

The novel isolation agar chromID EHEC supported the growth of all tested STEC and non-STEC *E. coli* strains, except for one STEC (O8:H9) and one non-STEC *E. coli* strain. However, the strains able to develop colonies showed significant reduced recovery percentages compared to TSA: 41% and 26%, respectively. The appearance on Chr ID did not distinguish STEC from non-STEC *E. coli* colonies - they were all purple (Figure III-1 b), with a few exceptions. Despite the supplementation of an unspecified cefixime-tellurite mix to Chr ID, no correlation could be observed between the presence of *terB* and the growth on this medium.

#### **4. Conclusions**

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The observed strong inhibition of Rapid *E. coli* O157:H7 towards STEC strains other than *E. coli* O157 makes the agar medium unsuitable for STEC isolation. Due to the high level of selectivity observed for Chr ST and mMac, most non-target organisms were sufficiently inhibited on these agars, which may facilitate the isolation of the major part of the tested STEC strains. Still, the isolation is limited to those strains that show resistance to the selective ingredients supplemented (e.g. tellurite, cefixime) to the chromogenic media. On the other hand, the biochemical characteristics (i.e. fermentative profile) of the different STEC strains are too diverse to use chromogenic media without supplementations. Therefore, the sole use of one of these two agars for the isolation of STEC from food might result in false negative. Paired use of two tested agar media might therefore be a useful option. The practical experience in this study with the more selective but easier to read Chr ST, in combination with a less selective agar like Chr ID or TBX, which allows the growth of all STEC strains, might be the best solution at present. However, in this study the observed colony colours and growth using pure cultures could only indicate the suitability of the media to support growth of the different STEC serotypes. In the presence of background micro-organisms or other STEC serotypes, adjacent colonies could influence the colony colour and growth of the target organisms (Kalchayanand *et al.*, 2013). In future research the isolation capabilities of combining two agars will be investigated using more complex food environment.

#### **Acknowledgements**

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## **Chapter 4: Evaluation of detection methods for non-O157 Shiga toxin-Producing *Escherichia coli* from food.**

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Adapted from:

Verhaegen B., Van Damme I., Heyndrickx M., Botteldoorn N., Elhadidy M., Verstraete K., Dierick K., Denayer S., De Zutter L., De Reu K. (2016) Evaluation of detection methods for non-O157 Shiga toxin-producing *Escherichia coli* from food. International Journal of Food Microbiology. 219, 64-70.

**Abstract**

Shiga toxin-producing *Escherichia coli* (STEC) remains a major foodborne pathogen of concern across the globe. Rapid detection and isolation of this pathogen is of great importance for public health reasons.

In this study the detection and isolation of four non-O157 STEC strains (O26, O103, O111, O145) from different artificially contaminated matrices, namely ground (minced) beef, cattle carcass swab, lettuce mix and sprouted soy beans, was evaluated. Low amounts of STEC were used (0.25 – 1.40 cfu/g) to spike the samples. All samples were enriched in parallel in Buffered Peptone Water (BPW) and Brila broth. After enrichment, detection was performed using real-time PCR (qPCR), and isolation using two chromogenic agar media, CHROMagar™ STEC and ChromID™ EHEC. Inoculation on the agar media was performed either directly after enrichment or after the use of an acid treatment procedure. Furthermore, the use of this procedure was also tested on naturally contaminated food products, using 150 *stx*-positive samples.

Although the qPCR Cycle Threshold (Ct) values were lower after enrichment in Brila broth, no significant differences in recovery were observed between both enrichment broths. Both agar media were equally suitable for the isolation of STEC, although a significantly higher recovery was obtained when using both agar media in parallel. For samples with a Ct value above 25, an acid treatment step prior to isolation ensured a significant improvement in the recovery of STEC due to the reduction in background microbiota. This acid treatment procedure proved especially useful for the isolation of STEC from sprouted soy bean samples.



## 1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC), remains a major foodborne pathogen of concern across the globe. In 2013, EFSA reported 6043 confirmed human cases in Europe, a notable proportion of which were caused by STEC serotype O157:H7, the most common serotype within the STEC group. However, the non-O157:H7 STEC serotypes are increasingly being recognized and reported as important foodborne pathogens. Still, in Europe the majority of STEC cases are sporadic cases (EFSA, 2015). Ruminants, especially cattle, are colonized by STEC and are regarded as the natural reservoir (Nataro and Kaper, 1998). Although animals infected with STEC normally show no signs of disease, it can be very pathogenic to humans, causing clinical symptoms ranging from mild to severe diarrhea, possibly complicated with hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Lorenz *et al.*, 2013). STEC can be transmitted to humans through many different routes, but mainly through consumption of contaminated foods, like raw or undercooked beef, raw milk, fecal contaminated water, fruits and vegetables. On the other hand person-to-person contact, or direct contact with animal feces or an animal reservoir can also be responsible (Jinneman *et al.*, 2012; Nataro and Kaper, 1998). The contamination of beef and fresh produce generally occurs during slaughter by fecal contamination of the cattle carcasses and the use of manure-based fertilizers or manure-contaminated water, respectively (Erickson and Doyle, 2007). Since modern dietary advice has begun promoting the consumption of leafy greens like lettuce and sprouted seeds (mostly consumed raw), an increasing number of outbreaks are associated with the consumption of these products (Hou *et al.*, 2013). This emphasizes the need for a rapid and sensitive method for the detection of this pathogen in different types of food products. However, various food matrices are complex environments with varying physico-chemical properties and interfering background microbiota. In the past, the effective detection method targeting *E. coli* O157:H7 resulted in the globally-used ISO standard 16654:2001 (ISO, 2001). It is very important to select a suitable enrichment and isolation medium, which should facilitate the STEC cells' growth while inhibiting the background microbiota and ensuring the isolation and confirmation of STEC from food (Baylis, 2008; Catarama *et al.*, 2003). Several methods have been attempted to eliminate the interfering background microbiota, such as the implementation of a post-enrichment immunomagnetic separation (IMS) procedure. While this procedure has proven effective for the isolation of *E. coli* O157, generally no significant effect was observed for non-O157 STEC (K Verstraete *et al.*, 2012). Another strategy to reduce the level of background microbiota is to use an acid treatment procedure. Acid treatment prior to plating on selective isolation media might be a rapid and economical alternative way to isolate STEC, especially for

STEC serogroups without commercially available IMS beads (Fedio *et al.*, 2012; Grant *et al.*, 2009; Tillman *et al.*, 2012).

In this study, we evaluated different detection and isolation procedures for STEC from food, using qPCR detection in combination with isolation using different enrichment and selective media. The selected enrichment and isolation media have been previously evaluated using pure cultures (Verhaegen *et al.*, 2015a, 2015b, e. i. chapter 2 & 3 ). Furthermore, the use of an acid treatment procedure prior to streaking onto the selective isolation media was compared to direct streaking for isolation of STEC from artificially and naturally contaminated food samples.

## **2. Material and methods**

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### **2.1. Strains**

STEC O26:H11 (MB 5316; *eae*, *stx1*); STEC O103:H2 (MB 5308; *eae*, *stx1*); STEC O111:H8 (MB 5310; *eae*, *stx1*, *stx2*) and STEC O145:H28 (MB 5850; *eae*, *stx2*) were used for artificial contamination of food samples. All strains were isolated from food samples and stored at -80°C using Pro-Lab Microbank cryovials (Pro-Lab, Ontario, Canada) according to the manufacturer's instructions.

### **2.2. Preparation of inoculums**

All strains were cultured onto Tryptone Soy Agar (TSA; Oxoid, Ltd., Basingstroke, Hampshire, England) plates at 37°C for 24h. These stock cultures were kept on TSA at 4°C and were renewed monthly. A single colony from these culture plates was transferred into Tryptone Soy Broth (TSB; Oxoid). After incubation at 37°C for 24h, the stationary phase cells were ten-fold serially diluted in TSB to obtain a concentration of approximately  $10^6$  cfu/ml. The inoculated TSB was stored in a refrigerator at 4°C for seven days to induce cold stress. After seven days, all cultures were individually diluted to a concentration of approximately 10 cfu/ml in Peptone Water (PW; Bio-Rad, Marnes-la-Coquettes, France) for inoculation of different food matrices, except the carcass swabs, which were inoculated with non-stressed STEC strains. The initial inoculum level was confirmed by plating in duplicate on TSA and incubation for 24 hrs at 37°C.

### **2.3. Preparation of artificially contaminated food matrices**

The experimental design is illustrated in Figure IV-1. For evaluation of the selected detection and isolation method, cattle carcass swab (n = 10), ground (minced) beef (n = 10), lettuce mix (n = 10) and sprouted soy beans (n = 10) samples were collected. All samples originated from different commercial batches purchased in several retail stores in Belgium. The carcass swabs were obtained from carcasses sampled during different sampling visits in one slaughterhouse, after

evisceration and trimming of the carcasses, but before cooling. For ground beef, lettuce mix and sprouted soy beans each sample was divided into 10 subsamples of 25 g in sterile polyethylene lateral filter bags (Gosselin, Borre, France). A swab sample of a cattle carcass consisted of five sponge swabs (3M, SSL100, St. Paul, MN, USA) each premoistened with 10 ml BPW and used to sample an area of approximately 625 cm<sup>2</sup> (= A4 format). Each carcass swab sample was diluted in 90 ml PW and homogenized by stomaching (Masticator, IUL S.A, Barcelona, Spain) for 2 minutes. This homogenized sample was divided in ten subsamples of 10 ml. For the artificial contamination, two subsamples per sample were inoculated with 1 ml of one of the four cultures (*E. coli* O26:H11, *E. coli* O103:H2, *E. coli* O111:H8, *E. coli* O145:H28). The final concentration was approximately 10 CFU per subsample. The remaining two subsamples were not inoculated and used as blank control samples.

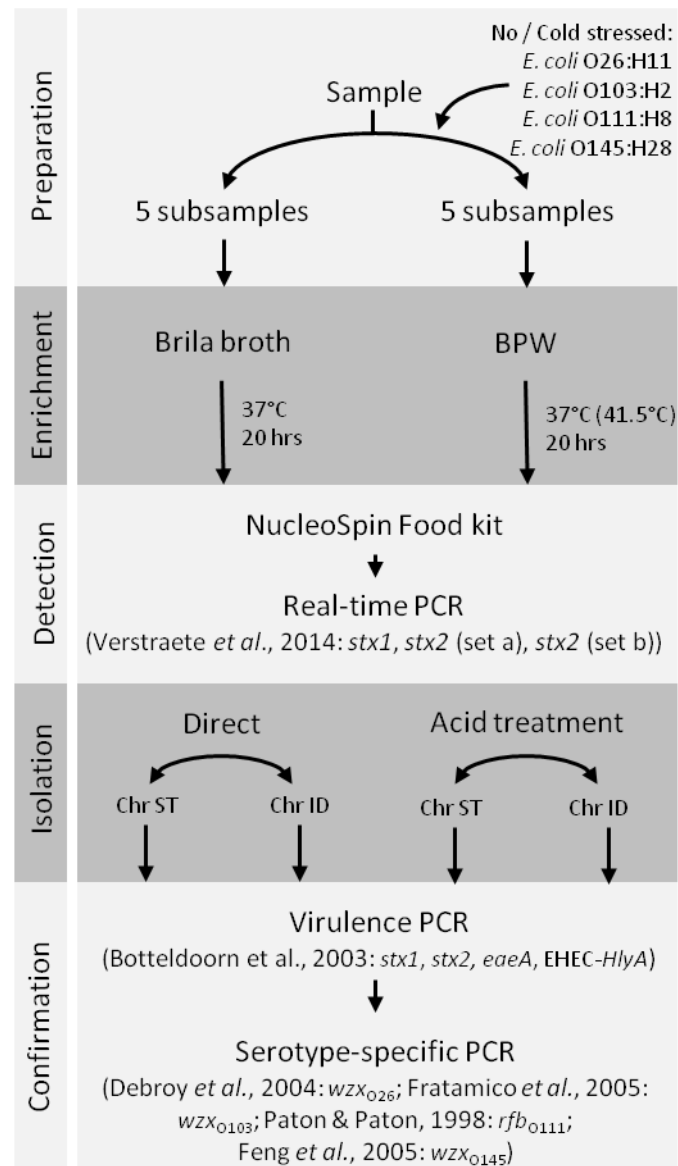


Figure IV-1. Flow chart showing the sample preparation and non-O157 STEC detection and isolation using enrichment media Brila broth or BPW followed by a DNA extraction step and real-time PCR detection. All enriched samples were plated either immediately or after acid treatment onto isolation media CHROMagar™ STEC (CHR ST) or ChromID™ EHEC (Chr ID) and confirmed using virulence –and serogroup-specific PCR.

#### **2.4. Enrichment**

Four of the subsamples each inoculated with one of the four cultures and one blank subsample were diluted to a 1/10 ratio using pre-warmed BPW (Buffered Peptone Water [Bio-Rad]) and the remaining subsamples using pre-warmed Brila broth (Merck), and all were homogenized by stomaching for 2 min. All enrichments were incubated at 37°C for 20 h, except the BPW enriched sprouted soy bean subsamples, which were incubated at 41.5°C for 20 h.

#### **2.5. Detection by real-time PCR**

A qPCR detection of STEC was carried out using 20 h enrichment broths. In this assay, three different primer sets are used in singleplex, one primer set for the detection of the subtypes of *stx1* and two primer sets for the detection of the subtypes of *stx2* (excluding *stx2f*). A qPCR for STEC detection targeting the virulence genes (*stx1* and *stx2*), was carried out using 20 h enrichment broths. One milliliter of each enriched broth was centrifuged for 10 minutes at 6,000 x g and genomic DNA (gDNA) was extracted from the pellet using the NucleoSpin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Finally the purified gDNA was stored at -20°C. All qPCR assays were performed on a LightCycler® 480 (Roche Diagnostics, Vilvoorde, Belgium). All products and protocol were as described by Verstraete *et al.* (2014). Briefly, the qPCR mixture (25 µl including 5 µl DNA template) contained: 1x TaqMan® Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA), 300nM of each primer and 100 nM of each TaqMan® probe (Eurogentec, Seraing, Belgium). Thermal protocols: initial incubation at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 1 min annealing and elongation at 60 °C, and a final cooling step at 40 °C for 30 s. The Cycle threshold (Ct) value was determined for each primer/probe set. In addition, all samples were spiked with TaqMan® exogenous internal positive control reagents (Life Technologies) to distinguish true target negatives from PCR inhibition.

#### **2.6. Isolation protocol**

Two isolation media were used during this study: CHROMagar™ STEC supplemented with 10 ml/l selective mix (CHROMagar Microbiology, Paris, France) and ChromID™ EHEC supplemented with 4 ml/l cefixime-tellurite mix (bioMérieux, Paris, France). The 20 h enriched broths were spread plated (10 µl) onto both isolation agar media. In parallel, acid treatment was carried out, followed by inoculation of 50 µl onto the two selective chromogenic agar media. The acid

treatment was based on the description by Fedio *et al.* (2012). Briefly, 2 ml of the 20 h enriched broth was centrifuged at 12,000 x g for 3 min. The pellet was resuspended in acidified TSB (pH = 2) and incubated on the rota-mix (DynaL, Invitrogen, Oslo, Norway) at room temperature for 30 min. The samples were again centrifuged at 12,000 x g for 3 min and the pellet was resuspended in 1 ml phosphate buffered saline (PBS, Oxoid) followed by plating (50 µl). All plates were incubated for 24 h at 37°C.

Following incubation, up to five suspect colonies from each inoculated plate were subcultured on TSA during 24 h at 37 °C. One colony of every subculture was transferred to 100 µl of sterile water and heated at 90 °C for 17 min. The lysed cells were transferred to our in-house PCR mixture and tested by a quadrumultiplex PCR method to confirm the presence of virulence genes as described by Botteldoorn *et al.* (2003), applying the primers for *stx1*, *eae*, and EHEC-*hlyA* described by Fagan *et al.* (1999) and for *stx2* described by Paton and Paton (1998). All positive isolates were further examined using a serogroup-specific PCR (O26: Debroy *et al.*, 2004; O103: Fratamico *et al.*, 2005; O111 Paton & Paton, 1998 and O145: Feng *et al.*, 2005).

## **2.7. Analysis of naturally contaminated food samples**

For the study of naturally contaminated food, 150 *stx*-positive food samples originating from the national monitoring plan were selected. This consisted of 63 cattle carcass swabs, 42 dairy samples, 40 meat samples and 5 vegetable samples. They were sampled by the food safety authorities (FASFC) in Belgium between February and June 2014. According to ISO/TS 13136:2012 portions of 25 g were added to 225 ml of BPW, homogenized and incubated at 37°C for 18-24 hrs. The enriched samples were screened for the presence of *eae* and *stx* genes using the DNA extraction kit (Extraction pack FOOD 1, Pall GeneDisc Technologies, Bruz, France) and GeneDisc multiplex PCR (Pall GeneDisc Technologies) according to the manufacturer's instructions. Further, the enriched samples were inoculated onto CHROMagar™ STEC, with and without prior described acid treatment procedure. Following an incubation period of 24 h at 37 °C, the suspected STEC colonies were confirmed by qPCR as described in the ISO/TS 13136:2012 protocol.

## **2.8. Statistical analysis**

The statistical analyses were performed in STATA/MP 12.1 (Stata Corporation, College Station, TX, USA). The comparison of the recovery (number of samples with isolation) obtained by the different detection methods were examined using a logistic regression analysis including matrix, sample number and serotype as random effect. The Ct-values of the two enrichment media were compared using a bootstrap median regression. The significance level of all analyses was set at 0.05.

### **3. Results**

The overall performance of the detection methods was determined by on the total number of samples inoculated with a low level of STEC strains that were positive by qPCR detection or isolation by culture plating. For each sample, a set of blank control subsamples was enriched and examined in the same way as the artificial inoculated samples. No STEC was detected or isolated from any of the control samples (data not shown).

#### **3.1. Enrichment and detection**

qPCR enabled detection of the typical virulence genes, *stx1* and *stx2* in the enriched samples. This detection was highly successful for all four food matrices after enrichment in Brila broth and BPW (Table IV-1). A 100% detection was obtained for ground beef, carcass swab and lettuce mix, except for sprouted soy bean, for which 36/40 (90%) and 35/40 (88%) positive samples were detected after enrichment in Brila broth and BPW, respectively. For this food matrix, strain variation was observed: especially the *E. coli* O145:H28 strain proved the most difficult to enrich to a detectable level. Furthermore, results of the TaqMan® exogenous internal positive control reagents showed no PCR inhibitions in any of the samples. The lowest Ct values for any of the *stx* primer/probe sets were considered in order to compare the growth during the 20 h of enrichment in Brila broth and BPW. The Ct values of samples enriched in Brila broth were significantly lower compared to BPW for all four matrices ( $P < 0.05$ ), except for ground beef ( $P > 0.05$ ) (Figure IV-2). In contrast, the total cultural isolation efficiency after 20 h of enrichment in either Brila broth or BPW yielded no statistical differences between both enrichment broths ( $P > 0.05$ ) (Figure IV-2). Notably, the Ct values of the *stx* genes for enriched sprouted soy bean samples were much higher than the other matrix samples.

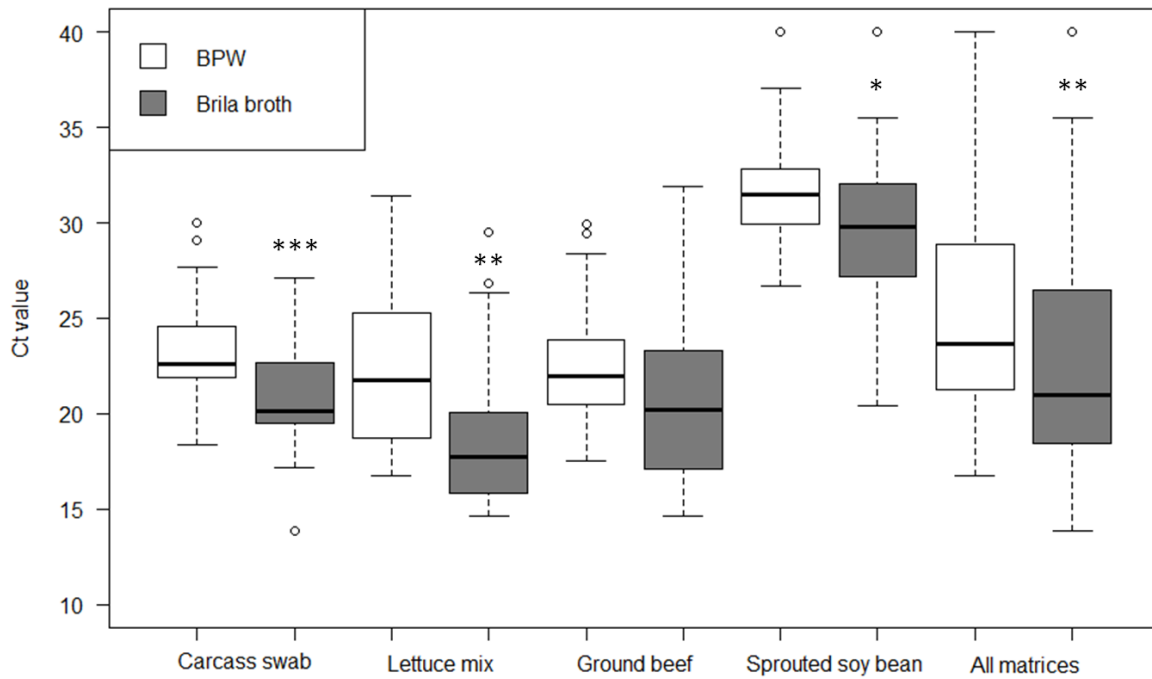


Figure IV-2. Box plot of the real-time PCR Ct values of *stx* after 20h enrichment in Brila broth or BPW, subdivided by matrix. Per matrix the significant difference between Brila broth and BPW was indicated by: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

### 3.2. Acid treatment procedure

The implementation of an acid treatment procedure during the cultural isolation of non-O157 STEC from the artificially inoculated food samples resulted in no statistical difference for cattle carcass swabs samples ( $P > 0.05$ ). On the other hand, the recovery of STEC was significantly higher after acid treatment for ground beef ( $P < 0.05$ ), sprouted soy bean ( $P < 0.0001$ ), and lettuce mix ( $P < 0.01$ ) (Table IV-1). The very low number of successful isolations without an acid treatment (1/40) from sprouted soy bean after enrichment in both media was significantly enhanced by acid treatment to 27/40 and 26/40 after enrichment in Brila broth and BPW, respectively. Moreover, three successful isolations were performed on BPW enriched sprouted soy bean samples that were negative for qPCR detection.

In lettuce mix the improvement was only observed after enrichment in BPW ( $P < 0.05$ ), but not with Brila broth. For the latter, a clear background microbiota reduction and more recognizable appearance of target colonies was observed in most replicates of all food matrices (Figure IV-3).

The combined use of both direct plating and acid treatment resulted in a significantly higher recovery compared to direct plating for all matrices ( $P < 0.05$ ), except for carcass swabs and lettuce mix samples after enrichment in BPW and Brila broth, respectively. However, this combined use of direct plating and acid treatment showed no significant difference in recovery as compared to the sole use of acid treatment for all matrices.

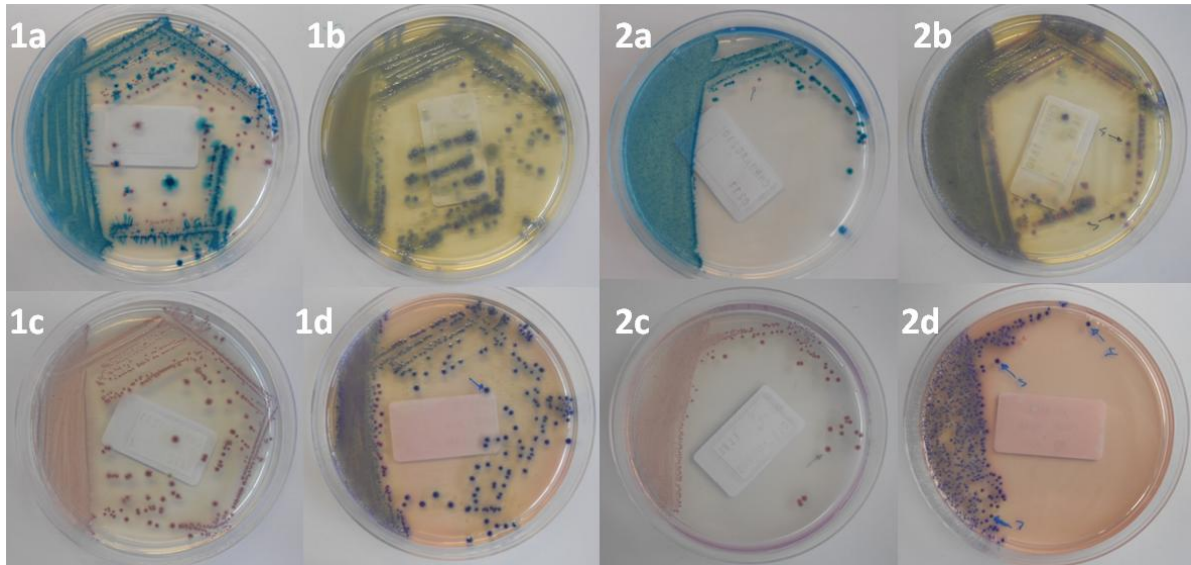


Figure IV-3. The recovery of STEC (MB 5316) on CHROMagar™ STEC (Chr ST; a,c) and ChromID™ EHEC (Chr ID; b,d), with (c,d) or without (a,b) prior acid treatment procedure from ground beef (1) and sprouted soy bean (2) samples after 20h enrichment in Brila.

The lowest Ct values for any of the *stx* primer/probe sets of each enriched sample in both Brila broth and BPW were considered to observe the cultural recovery percentages associated with the different Ct values. A clear difference was observed between recovery after direct plating and acid treatment when the Ct value was higher than a threshold set at 25 (Figure IV-4). All sample enrichment broths with a Ct < 25 showed no significant difference in recovery after direct plating or acid treatment for both Brila broth and BPW ( $P > 0.05$ ). In contrast, the samples that exceeded the 25 Ct threshold did show a significant higher recovery after acid treatment compared to direct plating for both Brila ( $P > 0.001$ ) and BPW ( $P > 0.001$ ) enriched broths.

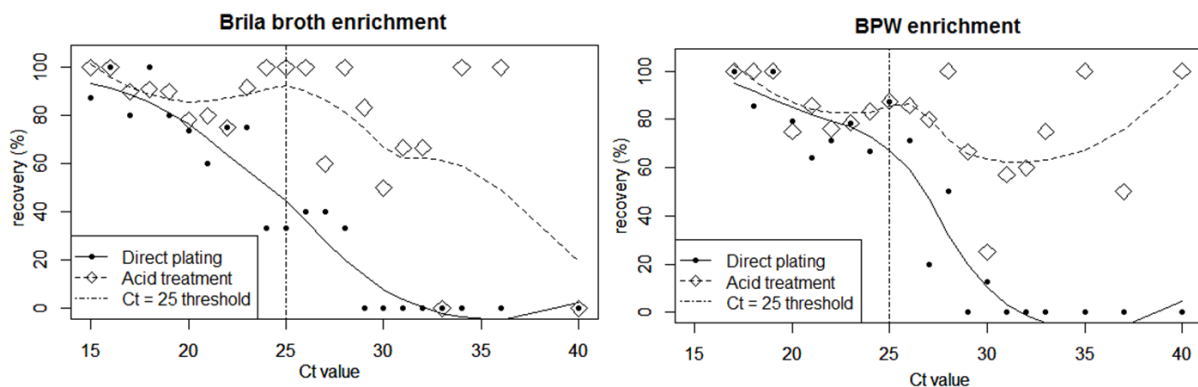


Figure IV-4. The recovery percentages after direct plating or acid treatment of the tested STEC strains from both BPW and Brila enrichment broths with different Real-Time PCR Ct values for *stx*. A Loess regression line was fitted to both isolation recovery from direct plating and acid treatment. A Ct > 25 for *stx* detection was selected as the threshold value from which the recovery after acid treatment was significantly different from direct plating ( $P < 0.001$ ).



### 3.3. *Cultural isolation*

In general, both isolation agar media showed some differences in number of successful isolations from the artificially contaminated samples (Table IV-2). The recovery of STEC after direct plating was higher using CHROMagar™ STEC compared to ChromID™ EHEC for ground beef and carcass swabs ( $P < 0.05$ ), but for lettuce mix ChromID™ EHEC showed a higher recovery (Table IV-1). Similarly, the recovery after acid treatment was higher using CHROMagar™ STEC for ground beef ( $P < 0.05$ ) and lower for lettuce mix ( $P < 0.05$ ). The parallel use of both chromogenic media significantly increased the recovery compared to the use of one medium (Table IV-2).

All tested STEC strains grew on CHROMagar™ STEC and ChromID™ EHEC as distinctive mauve and purple colonies, respectively. Characteristic colonies on CHROMagar™ STEC could more easily be differentiated from non-target organisms whereas suspect colonies on ChromID™ EHEC could more often not be confirmed as positive (data not shown). In general, a clear strain variation was observed. While the *E. coli* O26:H11 strain could be isolated in most cases and on both isolation media, the *E. coli* O103:H2 strain could rarely be isolated on CHROMagar™ STEC and often on ChromID™ EHEC (Table IV-2).

To further compare the efficiency of the isolation of STEC from food with and without an acid treatment procedure, 150 naturally contaminated food samples positive for *stx* in the qPCR screening were analyzed. A summary of results for the untreated and acid-treated inoculations on CHROMagar™ STEC is shown in Table IV-3. The recovery of STEC showed no significant difference with or without an acid treatment procedure ( $P > 0.05$ ) but when combining both techniques the recovery was significantly better compared to either untreated or acid-treated inoculations ( $P < 0.05$ ). In vegetables, no STEC isolation could be obtained.

Table IV-1. Real time-PCR detections (qPCR) of non-O157 STEC strains from artificially contaminated ground beef, cattle carcass swabs, sprouted soy bean and lettuce mix sample after enrichment in Brila broth or BPW. Cultural isolations were performed onto isolation media (CHROMagar™ STEC (CHR ST) or ChromID™ EHEC (Chr ID)) using streaking performed either immediately or after acid treatment. <sup>1</sup>Per food matrix, the totals with a different superscript letter are significantly different ( $P < 0.05$ ).

Matrix	STEC serotype	inoculum (cfu/gram)	n	qPCR		Cultural isolation					
				Brila	BPW	Brila broth			BPW		
						Direct	Acid	Combined <sup>***</sup>	Direct	Acid	Combined
Ground beef	O26:H11	0.57 ± 0.45	10	10*	10	8**	10	10	9	9	9
	O103:H2	0.78 ± 0.18	10	10	10	5	7	7	5	7	9
	O111:H8	0.53 ± 0.47	10	10	10	6	7	8	6	9	9
	O145:H2	0.38 ± 0.19	10	10	10	7	8	8	5	7	7
	Total <sup>1</sup>		40	40 (100%)	40 (100%)	26 (65%) <sup>A</sup>	32 (80%) <sup>B</sup>	33 (83%) <sup>B</sup>	25 (63%) <sup>A</sup>	32 (80%) <sup>B</sup>	34 (85%) <sup>B</sup>
Cattle carcass swab	O26:H11	0.38 ± 0.02	10	10	10	8	10	10	8	8	8
	O103:H2	0.60 ± 0.05	10	10	10	2	4	5	0	4	4
	O111:H8	0.52 ± 0.14	10	10	10	9	10	10	10	9	10
	O145:H2	0.75 ± 0.11	10	10	10	10	9	10	9	6	9
	Total <sup>1</sup>		40	40 (100%)	40 (100%)	29 (73%) <sup>A</sup>	33 (83%) <sup>CD</sup>	35 (88%) <sup>BD</sup>	27 (68%) <sup>A</sup>	27 (68%) <sup>A</sup>	31 (78%) <sup>AD</sup>
Sprouted soy bean	O26:H11	0.25 ± 0.13	10	9	10	0	9	9	0	10	10
	O103:H2	0.50 ± 0.21	10	9	10	0	3	3	0	4	4
	O111:H8	0.33 ± 0.10	10	10	10	1	10	10	1	7	8
	O145:H2	0.25 ± 0.24	10	8	5	0	5	5	0	5	5
	Total <sup>1</sup>		40	36 (90%)	35 (87.7%)	1 (3%) <sup>A</sup>	27 (68%) <sup>B</sup>	27 (68%) <sup>B</sup>	1 (3%) <sup>A</sup>	26 (65%) <sup>B</sup>	27 (68%) <sup>B</sup>
Lettuce mix	O26:H11	0.75 ± 0.27	10	10	10	10	10	10	9	10	10
	O103:H2	1.40 ± 0.59	10	10	10	10	10	10	9	10	10
	O111:H8	1.10 ± 0.16	10	10	10	10	10	10	8	10	10
	O145:H2	0.23 ± 0.15	10	10	10	8	9	10	3	9	9
	Total <sup>1</sup>		40	40 (100%)	40 (100%)	38 (95%) <sup>A</sup>	39 (98%) <sup>A</sup>	40 (100%) <sup>A</sup>	29 (73%) <sup>BC</sup>	39 (98%) <sup>A</sup>	39 (98%) <sup>A</sup>

\* Number of successful RT-PCR detections of *stx* out of a total number of samples *n*, \*\* Number of successful cultural isolations out of a total number of samples *n*, \*\*\* Results of the combined use of both direct plating and plating after acid treatment

Table IV-2. Isolation of non-O157 STEC strains from the artificially contaminated ground beef, cattle carcass swabs, sprouted soy bean and lettuce mix sample after enrichment (Brila or BPW). Inoculations were performed onto isolation media (CHROMagar™ STEC (Chr ST) or ChromID™ EHEC (Chr ID)) using either streaking performed immediately or after acid treatment. <sup>1</sup>Per food matrix, the totals with a different superscript letter are significantly different ( $P < 0.05$ ).

Matrix	STEC serotype	<i>n</i>	Brila broth						BPW					
			Direct			Acid			Direct			Acid		
			Chr ST	Chr ID	Combined**	Chr ST	Chr ID	Combined	Chr ST	Chr ID	Combined	Chr ST	Chr ID	Combined
Ground beef	O26:H11	10	8*	5	8	10	6	10	9	5	9	9	4	9
	O103:H2	10	1	4	5	3	7	7	1	4	5	0	7	7
	O111:H8	10	5	3	6	7	4	7	5	4	6	9	5	9
	O145:H28	10	7	2	7	8	3	8	5	3	5	7	2	7
	Total <sup>1</sup>	40	21 <sup>AD</sup>	14 <sup>B</sup>	26 <sup>A</sup> EH	28 <sup>CEG</sup>	20 <sup>ABD</sup>	32 <sup>EF</sup>	20 <sup>ADH</sup>	16 <sup>BD</sup>	25 <sup>AGH</sup>	25 <sup>A</sup> EH	18 <sup>BD</sup>	32 <sup>CF</sup>
Cattle carcass swab	O26:H11	10	8	3	8	10	4	10	8	2	8	7	4	8
	O103:H2	10	0	2	2	0	4	4	0	0	0	0	4	4
	O111:H8	10	8	7	9	8	9	10	9	7	10	7	8	9
	O145:H28	10	9	5	10	9	8	9	7	3	9	4	5	6
	Total <sup>1</sup>	40	25 <sup>A</sup> F	17 <sup>BC</sup>	29 <sup>FD</sup>	27 <sup>A</sup> F	25 <sup>A</sup> F	33 <sup>D</sup>	24 <sup>A</sup> EE	12 <sup>B</sup>	27 <sup>A</sup> F	18 <sup>BE</sup>	21 <sup>A</sup> CE	27 <sup>A</sup> F
Sprouted soy bean	O26:H11	10	0	0	0	9	4	9	0	0	0	10	4	10
	O103:H2	10	0	0	0	0	3	3	0	0	0	0	4	4
	O111:H8	10	0	1	1	9	8	10	0	1	1	2	6	7
	O145:H28	10	0	0	0	5	2	5	0	0	0	4	2	5
	Total <sup>1</sup>	40	0 <sup>A</sup>	1 <sup>A</sup>	1 <sup>A</sup>	23 <sup>BC</sup>	17 <sup>B</sup>	27 <sup>C</sup>	0 <sup>ABC</sup>	1 <sup>A</sup>	1 <sup>A</sup>	16 <sup>B</sup>	16 <sup>BD</sup>	26 <sup>CD</sup>
Lettuce mix	O26:H11	10	10	9	10	10	9	10	7	8	9	9	9	10
	O103:H2	10	1	10	10	1	10	10	0	9	9	2	10	10
	O111:H8	10	8	10	10	9	10	10	3	8	8	10	10	10
	O145:H28	10	6	5	8	9	8	9	2	2	3	9	7	9
	Total <sup>1</sup>	40	25 <sup>AG</sup>	34 <sup>BD</sup>	38 <sup>DE</sup>	29 <sup>AB</sup>	37 <sup>DE</sup>	39 <sup>CE</sup>	12 <sup>F</sup>	27 <sup>A</sup>	29 <sup>ABG</sup>	30 <sup>AB</sup>	35 <sup>DE</sup>	39 <sup>E</sup>
			(63%)	(85%)	(95%)	(73%)	(92%)	(98%)	(30%)	(68%)	(73%)	(5%)	(90%)	(98%)

\* Number of successful cultural isolations out of a total number of samples *n* \*\* Results of the combined use of both Chr ST and Chr

ID

Table IV-3. Cultural isolations of STEC from naturally contaminated food, consisting of dairy products, meat products, vegetables and cattle carcass swabs, after 18- 24 h of enrichment in BPW. Inoculations were performed onto isolation media (CHROMagar™ STEC (CHR ST)) using streaking performed either immediately or after acid treatment. Totals with a different superscript letter indicates a significant difference ( $P < 0.05$ ).

Matrix	Number of <i>stx</i> -positive samples	Cultural isolation		
		Direct	Acid	Combined**
Dairy samples	42	7 <sup>a</sup>	5	10
Meat samples	40	5	6	7
Carcass swabs	63	9	7	12
Vegetables	5	0	0	0
Total	150	21 (14%) <sup>A</sup>	18 (12%) <sup>A</sup>	29 (19%) <sup>B</sup>

\* Number of successful cultural isolations out of the total number of *stx*-positive samples in screening, \*\* Results of the combined use of both direct plating and plating after acid treatment

#### 4. Discussion

Because STEC present at low levels in foods can cause serious foodborne illness, detection methods sensitive enough to identify only few STEC cells in food matrices are needed. Therefore, the enrichment as first step in any protocol for STEC detection and isolation is crucial to ensure a rapid growth to a detectable level. Currently, the International Organization for Standardization (ISO) recommends the use of buffered peptone water (BPW) as enrichment medium when the bacteria may have undergone stress conditions (ISO, 2012). Verhaegen *et al.* (2015b) evaluated several enrichment media using pure STEC cultures and confirmed better growth dynamics in BPW compared to more selective enrichment media, such as Brila broth (e. i. chapter 2). However, in the present study the qPCR Ct values of artificially inoculated food samples enriched in Brila broth were significantly lower compared to BPW for all four matrices, indicating a better growth of the STEC cells during enrichment in Brila broth. The latter medium contains brilliant green and bile salts, which inhibit the growth of Gram-positive bacteria. Tzschoppe *et al.* (2012) has also shown that BPW is less inhibitory against natural background microbiota compared to Brila broth for the detection and isolation of STEC from lettuce and sprouted seeds. We also observed an improved recovery rate from lettuce mix after enrichment in Brila broth. However, the introduction of an acid treatment procedure as a way to reduce the background microbiota proved to be sufficient to ensure a equally high recovery rate after BPW enrichment. For sprouted soy bean, no improved isolation was noted after enrichment in Brila broth compared to BPW. Furthermore, remarkably high qPCR Ct values from sprouted soy bean samples were observed with both enrichment media, which indicates that STEC grew less

efficiently in this matrix, which is known to contain high numbers of interfering background microbiota (Fedio *et al.*, 2012). Moreover, in several artificially contaminated samples STEC was unable to grow to a detectable level, resulting in false negative results.

The use of IMS for the isolation of *E. coli* O157:H7 is an effective tool to isolate the target organism from samples with interfering background microbiota. However, in the presence of a high number of background microbiota a significant portion of non-target-organisms might be carried over during the IMS protocol and interfere during isolation on agar media. One of the strategies to reduce this interference is the implementation of an acid treatment to eliminate these organisms from the IMS beads (Fedio *et al.*, 2012; Yoshitomi *et al.*, 2012). This technique is based on an important feature of *E. coli*, namely its tolerance to extremely acidic conditions (Bhagwat *et al.*, 2005; Grant, 2004). While IMS was proven less effective for the isolation of non-O157 STEC than for *E. coli* O157:H7 (Verstraete *et al.*, 2010), Tillman *et al.* (2012) demonstrated that the combination of a selective chromogenic agar medium with post-IMS acid treatment increases the likelihood of isolating non-O157 STEC strains. They also reported the acid sensitivity of non-*E. coli*, such as *Enterobacter* spp., *Klebsiella* spp. and *Citrobacter* spp. In the present study, acid treatment (used without IMS) was most effective for the isolation of non-O157 STEC from sprouted soy bean, which is similar to the findings by Fedio *et al.* (2012) for the isolation of STEC O157:H7 from this matrix. Using acid treatment, we obtained higher recovery rates from sprouted soy bean compared to the recovery from sprouted seeds described by Verstraete *et al.* (2012), where fewer successful isolations were obtained even using a higher artificial inoculation level. The increased recovery could be explained, besides the acid tolerance of *E. coli*, by the up-concentration of the inoculum during the acid treatment step and the 5-fold increase in inoculum volume compared to direct plating. We found that isolation of STEC from enriched samples yielding a qPCR Ct value > 25 for detection of *stx* is more successful after acid treatment. Those high Ct values indicate a lower concentration of STEC after enrichment (a Ct value of 25 corresponds to approximately 4 log of pure STEC genomic DNA copies in control reactions). For naturally-contaminated food samples, however, only the combination of both techniques resulted in a significant improved isolation rate, confirming the usefulness of the acid treatment. A broad variety of STEC strains might be present in these naturally contaminated samples with different biochemical characteristics (i.e., fermentative profile) and antibiotic sensitivities, compared with the four STEC strains used for the artificially contaminated samples. Furthermore, for the isolation from the naturally contaminated samples only one isolation medium, CHROMagar™ STEC, was used in this study.

As shown in multiple studies, CHROMagar™ STEC is a valuable selective isolation medium, although its use is limited to those STEC strains with a resistance for the selective ingredients,

such as potassium tellurite (Gill *et al.*, 2014; Kase *et al.*, 2015; Tzschoppe *et al.*, 2012; Verhaegen *et al.*, 2015a (e. i. chapter 3)). Also in our study, notwithstanding the growth inhibition of one of the tested strains (STEC O103:H2), CHROMagar™ STEC resulted in a higher recovery rate compared to ChromID™ EHEC. Higher recovery was only found for lettuce mix samples when using ChromID™ EHEC. While this might be explained by the particular composition of the interfering background microbiota accompanying lettuce mix samples, the exact explanation of this finding remains unclear. Not all STEC strains are able to grow on CHROMagar™ STEC and recovery might be dependent on the food matrix. Therefore the paired use of highly selective with a second less selective isolation medium, as shown in this study, increases the STEC recovery.

The chance of recovery of STEC on a low-selective isolation medium, such as ChromID™ EHEC, or the ISO/TS 13136:2012 suggested Tryptone Bile X-glucuronide agar (TBX) by random picking of suspect colonies is rather low (Cooley *et al.*, 2013). Using these media the confirmation remains a labor-intensive and time-consuming practice of isolating multiple presumptive positive colonies (up to 50), followed by colony pooling and confirming by (q)PCR. In the present study only a limited number of colonies (up to five) per inoculated plate were selected for confirmation tests. For this reason the recovery rates might have been even higher if more colonies were tested.

## **5. Conclusion**

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A rapid and robust detection of STEC from food is of utmost importance to ensure appropriate actions to safeguard public health. Therefore, the authors suggest an improved method for detection and isolation of low levels of STEC. Enrichment in either BPW or more-selective Brila broth resulted in most cases in detectable levels of STEC through qPCR (*stx* gene) or isolation. While lower qPCR Ct values for *stx* detection were observed for samples enriched in Brila broth compared to BPW, this did not result in significant differences between isolation rates between both enrichment media. For enriched samples with Ct values > 25 for *stx* detection, the authors advise the use of an additional acid treatment step on the bacterial pellet before isolation. Furthermore, all isolations should preferably be performed using both a selective and less-selective isolation medium, such as CHROMagar™ STEC and ChromID™ EHEC, which are based on different biochemical principles.

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## **Chapter 5: Characteristics and virulence profile of Shiga toxin-Producing *Escherichia coli* isolated from food in Belgium**

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Verhaegen B., Barbau-Piednoir E., De Reu K., De Zutter L., Piérard D., Van Damme I., Denayer S., De Keersmaecker S., Heyndrickx M.. Characteristics and virulence profile of Shiga toxin-Producing *Escherichia coli* isolated from food in Belgium

## Abstract

Shiga toxin-producing *Escherichia coli* (STEC) remains a foodborne pathogen of major concern. As both low-pathogenic and highly pathogenic STEC strains produce Stx and are frequently recovered from food, assessing the pathogenic potential of strains remains of utmost importance. In this study, we characterized a large collection of STEC isolates from food (n=242) and human patients (n=40) in Belgium, in addition to 46 non-STEC *E. coli* isolates from food. For this purpose, the serotype and pathotype were investigated, in addition to the genetic relatedness as determined by the combined data of pulsed-field gel electrophoresis (PFGE) and genetic virulence profile.

Of the 328 isolates, 251 (77%) were AE-STEC (*eae*<sup>+</sup> STEC), 31 (9%) were STEC (*eae*<sup>-</sup> STEC), 35 (11%) were aEPEC (atypical EPEC) and 11 (3%) were negative for all tested pathotype specific genes. Non-LEE (NLE) genes (OI-122; *ent/espL2*, *nleE* and OI-71; *nleA*, *nleF* and *nleH1-2*) and the plasmid encoded genes (*ehxA*, *katA*) were more often detected in AE-STEC compared to STEC. Moreover, the NLE genes (except *nleA*) were more frequently detected in AE-STEC isolated from human patients compared to those from food origin. The remaining two plasmid encoded genes (*saa*, *subA*) were rarely detected. The analysis of these virulence profiles combined with the PFGE patterns revealed a number of pulsogroups. We observed that certain pulsogroups contain isolates with a potentially higher pathogenicity potential; these pulsogroups also included the human isolates and are therefore of public health importance. When in the future strains are isolated from food, their virulence potential could be estimated using the described characterization method and pulsogroups in this study.

## 1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxin-producing *E. coli* (VTEC), was first recognized as a zoonotic pathogen during outbreaks occurring in 1982 (Kaper and O'Brien, 2014). *E. coli* O157:H7 was isolated from patients developing hemorrhagic colitis (HC). Today, serotype O157:H7 remains the STEC serotype most frequently associated with severe symptoms, such as bloody diarrhea, hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP). Therefore, most studies have examined the epidemiology and pathogenesis of *E. coli* O157:H7. However, due to the intensified and improved STEC detection in patients, animals and food, an increased number of *E. coli* O157 but also non-O157 STEC strains were isolated in the past decade. *E. coli* of almost all known serogroups were found associated with Stx production (Beutin and Fach, 2014; EFSA, 2015). In Europe, the most frequently isolated non-O157 STEC serogroups belong to O26, O91, O103, O111, and O145 (EFSA, 2015; Mainil and Daube, 2005; Wang *et al.*, 2013). Since most human infection with STEC are food-borne via consumption of contaminated meat, milk, fruits, vegetables or water (Caprioli *et al.*, 2005), it is important to assess the public health risks associated with STEC strains commonly isolated from food. Therefore, we characterized a large part of the Belgian collection of STEC isolates from food, collected over a period from 2000 to 2014. While Stx production is the primary virulence trait responsible for severe symptoms, many STEC strains do not cause any symptoms. Therefore, assessing the potential clinical and public health risks associated with different STEC strains remains of utmost importance (Karmali *et al.*, 2003). Human pathogenic STEC strains often possess intimin, an outer membrane protein encoded by the *eae* gene responsible for “Attaching and Effacing” (A/E) lesions on epithelial cells. In addition, these strains often harbor a large virulence plasmid and induce clinical symptoms such as HC and HUS. Therefore, the classification as “enterohemorrhagic *E. coli*” (EHEC) was devised to denote these particular pathogenic STEC strains. However, as several authors have deviated from the definition of EHEC in the past, confusion arose in the scientific literature (Nataro and Kaper, 1998). Therefore, Pierard *et al.* (2012) suggested to disregard the term EHEC and proposed to include virulence-associated properties into the nomenclature, in this case AE-STEC for those STEC strains producing A/E lesions. This nomenclature will be followed in this study. The proposed nomenclature fits perfectly to the scheme developed by Karmali *et al.* (2003), in which STEC is classified into five “seropathotypes” (A to E) based on the combination of serotype with the severity of caused symptoms. However, this valuable scheme is limited to the described serotypes and their virulence potential. Moreover, in some cases strains belonging to the most virulent seropathotype A have been isolated from healthy humans. Hence, it is postulated that many other virulence and adherence factors must be involved in the pathogenicity of STEC (Karmali *et*

*et al.*, 2003; Scheutz, 2014). These factors are most often encoded by genes located on mobile genetic elements, such as plasmids, bacteriophages, transposons and pathogenicity islands (PAI). The latter is a class of genomic islands present on the genomes of pathogenic strains but absent from the genomes of non-pathogenic members of the same or related species (Hacker and Kaper, 2010). The best known PAI for STEC is the locus of enterocyte effacement (LEE), that harbors the *eae* gene and encodes the type III secretion system necessary for the typical A/E lesions. In recent years many non-LEE-encoded effector (NLE) genes associated with virulence and severe disease in humans were identified. These include *ent/espL2* and *nleE* that are encoded on the second most characterized PAI, namely the O-Island 122 (OI-122). Other PAI have been described, such as OI-71, OI-57, OI-43/48 and OI-36 (Coombes *et al.*, 2008; Ju *et al.*, 2014). Furthermore, plasmids may carry additional virulence and adherence factors such as enterohemolysin (*ehxA*), STEC auto-agglutinating adhesion factor (*saa*), subtilase cytotoxin (*subA*) and catalase-peroxidase (*katP*) (Brunder *et al.*, 1999; Paton and Paton, 2002; Paton *et al.*, 2004).

The presence of these virulence genes was investigated in the collection of food isolates included in this study. The resulting virulence profile combined with the pulsed-field gel electrophoresis (PFGE) data was used to determine the genetic relatedness. Furthermore, the same analyses were performed on STEC isolates from patients in order to explore similarities between food and human isolates, and identify relevant virulence factors.

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## **2. Materials and Methods**

### **2.1. *E. coli* strains**

A total of 328 isolates were analyzed in this study. These strains were isolated from food (n=288; meat n=67, cattle carcass swab n=176, dairy n=27, vegetables n=2, food type not reported n=16) and human faeces (n=40). The food isolates were part of the collection of the national reference laboratory (NRL) VTEC-food at the Scientific Institute of Public Health (WIV-ISP) in Belgium, which contained approximately 400 isolates and were isolated over a period from 2000 to 2014. The human isolates were part of the collection of the national reference centre (NRC) for pathogenic *E. coli* at UZ-VUB Brussels in Belgium. These isolates (STEC O26 n=5, STEC O103 n=5, STEC O111 n=5, STEC O145 n=5 and STEC O157 n=20) were randomly selected, independent of clinical symptoms, from the collection of strains isolated in the period January 2011 to September 2013.

## 2.2. Genetic characterization

Five multiplex PCRs were performed and three Luminex xMAP assays (developed at WIV-ISP, publication in preparation) to identify the clinically most relevant O-type (O26, O45, O55, O91, O103, O104, O111, O113, O118, O121, O128, O145, O157), H-Type (H2, H7, H8, H11, H19, H21, H28), pathotype specific genes (*eae*, *stx1*, *stx2* (including *stx2f*, *bfpA*, *elt*, *estA1*, *estA2-4*, *ipaH*, *aggR*) and resistance gene (*terB*). Moreover, virulence genes residing in PAI were identified (OI-122: *ent/espL2*, *nleE*, OI-71: *nleA*, *nleF*, *nleH1-2*) and virulence genes residing in plasmids (*katP*, *ehxA*, *saa*, *subA*).

## 2.3. Determination of *E. coli* pathotype

The genotype based on the genes *stx1*, *stx2*, *eae*, *bfpA*, *elt*, *estA1*, *estA2-4*, *ipaH* and *aggR* was used to define the diarrheagenic *E. coli* pathotype: typical enteropathogenic *E. coli* (tEPEC; *eae*+, *bfpA*+), atypical EPEC (aEPEC; *eae*+, *bfpA*-), STEC (*stx1*+ and/or *stx2*+), AE-STECS (*eae*+, *stx1*+ and/or *stx2*+), enteroaggregative *E. coli* (*aggR*+), enterotoxigenic *E. coli* (*elt* (Heat Labile Toxin)+ and/or *estA1* (Heat Stable Toxin variant h (STh))+ and/or *estA2-4* (Heat Stable Toxin variant p (STp))+) and enteroinvasive *E. coli* (*ipaH*+). Those isolates with no detectable pathotype specific genes were termed apathogenic *E. coli* (EC) in this study.

## 2.4. Pulsed-field gel electrophoreses (PFGE)

The plugs were prepared and digested with *XbaI* and separated by PFGE according to the PulseNet protocol for *E. coli* O157:H7 (<http://www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf>). The PFGE gel images were visually analyzed with BioNumerics version 7.5 (Applied Maths, Sint-Martens-Latem, Belgium) using *Salmonella* Braenderup H9812 as a normalization reference. The similarities between PFGE patterns and virulence profiles (*eae*, *stx1*, *stx2*, *exxA*, *saa*, *subA*, *katP*, *ent/espL2*, *nleE*, *nleA*, *nleF* and *nleH1-2*) were calculated using the Dice coefficient (with an optimization of 1% and a position tolerance of 1% for PFGE patterns), and were used to group the isolates according to their similarities using UPGMA (Unweighted pair-group method with arithmetic mean). Both analyses were combined based on average values. Pulsogroups were delineated on the basis of 80% similarity according to Dice similarity. Isolates that were not found within a group at 80% similarity, were considered as single isolates. Pulsosubgroups were delineated on the basis of 90% similarity.

## **2.5. Statistical analyses**

The statistical analyses were performed in STATA/MP 12.1 (Stata Corporation, College Station, TX, USA). Differences in frequencies of target genes (denoted in binary values 0 and 1) between the groups (AE-STE<sub>C</sub>, STE<sub>C</sub>, EPEC, EC) and between the source (human and food) were determined using a logistic regression analysis with Bonferroni corrections. The significance level of all analyses was set at 0.05

## **3. Results**

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### **3.1. Pathotypes**

Out of the total of 328 isolates, 46 (14%) were negative for both *stx1* and *stx2*. In eleven of these isolates no other pathotype specific genes were identified (apathogenic *E. coli*; Table V-1). The remaining 35 *stx*-negative isolates (11%) were positive for the *eae* gene and were classified as aEPEC, since none of these harbored an additional *bfpA* gene. The remaining 282 isolates (86%) were positive for *stx* (for the 242 food isolates: *stx1*<sup>+</sup> 50, *stx2*<sup>+</sup> 144, *stx1*<sup>+</sup> and 2<sup>+</sup> 48 and for the 40 human isolates: *stx1*<sup>+</sup> 14, *stx2*<sup>+</sup> 17, *stx1*<sup>+</sup> and 2<sup>+</sup> 9).

Table V-1. Prevalence of virulence genes in both human and food isolates grouped in *eae*-positive STEC (AE-STEC), *eae*-negative STEC (STEC), enteropathogenic *E. coli* (EPEC) and by this study termed apathogenic *E. coli* (EC). In addition the distribution of the virulence genes is displayed for the most common serotypes.

Plasmid												O1-122				O1-71						
		<i>ehxA</i>	<i>saa</i>	<i>subA</i>	<i>katP</i>	<i>ent/espL2</i>	<i>nleE</i>	<i>nleA</i>	<i>nleH1-2</i>	<i>nleF</i>	<i>terB</i>											
AE-STECC	Human	(n=39) <sup>1</sup>	39	100%	0	0%	0	32	82%	38	97%	38	97%	30	77%	39	100%	39	100%			
		(n=212)	182	86%	1	0.50%	0	0%	180	85%	145	68%	133	63%	112	53%	144	68%	125	59%	160	75%
		O157:H7 (n=149)	129	87%	1	1%	0	0%	147	99%	108	72%	92	62%	85	57%	96	64%	92	62%	111	74%
		O26:H11 (n=16)	13	81%	0	0%	0	0%	12	75%	15	94%	12	75%	12	75%	12	75%	16	100%	15	94%
		O103:H2 (n=11)	9	82%	0	0%	0	0%	7	64%	6	55%	10	91%	0	0%	11	100%	2	18%	4	36%
		O145:H28 (n=7)	7	100%	0	0%	0	0%	6	86%	7	100%	6	86%	0	0%	6	86%	1	14%	7	100%
		O111:H8 (n=5)	4	80%	0	0%	0	0%	5	100%	2	40%	5	100%	1	20%	5	100%	5	100%	5	100%
STEC	Human	(n=1) <sup>2</sup>	1	*	0	*	0	*	0	*	1	*	1	*	0	*	1	*	0	*	0	*
		(n=30)	15	50%	2	7%	0	0%	7	23%	3	10%	3	10%	1	3%	3	10%	2	7%	19	63%
		O157:H7 (n=1)	1	*	0	*	0	*	1	*	1	*	1	*	1	*	1	*	1	*	1	*
		O145:H28 (n=1)	0	*	0	*	0	*	0	*	1	*	1	*	0	*	1	*	0	*	1	0
EPEC		(n=35)	26	75%	1	2.90%	0	0%	23	66%	29	83%	25	71%	22	63%	26	74%	24	69%	33	94%
		O157:H7 (n=19)	18	95%	0	0%	0	0%	19	100%	19	100%	16	84%	12	63%	16	84%	14	74%	19	100%
		O26:H11 (n=7)	2	29%	0	0%	0	0%	2	29%	6	86%	4	57%	3	43%	5	71%	7	100%	6	86%
		O145:H28 (n=2)	2	100%	0	0%	0	0%	2	100%	2	100%	2	100%	1	50%	1	50%	1	50%	2	100%
EC	Food	(n=11)	2	18%	0	0%	0	0%	2	18%	0	0%	1	9%	0	0%	0	0%	0	0%	2	18%

\*No percentage was calculated, since the group contained only one isolate. <sup>1</sup>Human *E. coli* isolates consisting of 20 O157, 5 O26, 4 O103, 5 O111 and 5 O145 serogroups, <sup>2</sup>Single human STEC isolate of serogroup O103

### 3.2. Serotypes

In this study 13 different O-types (LPS) and 7 different H-types (Flagel) were identified in the 288 food isolates. The most prevalent STEC serotypes in food were *E. coli* O157:H7, O26:H11, O103:H2 and O145:H28 with 150 (62%), 16 (6.6%), 12 (5%) and 8 (3%) isolates, respectively. Serotype O111:H8 contained 5 isolates and four serotypes contained only one isolate (O157:H21, O113:H21, O113:H11, O104:H2). Twenty-six isolates were untypeable (NT) for both O- and H-type. For eight isolates only an O-type was identified (1 O118:HNT, 1 O103:HNT, 1 O55:HNT, 1 O104:HNT, 2 O113:HNT). For 15 isolates only an H-type was identified (7 ONT:H21, 3 ONT:H2, 3 ONT:H28, 1 ONT:H8, 1 ONT:H7). The most prevalent aEPEC serotypes in food were again O157:H7 and O26:H11 with 19 (54%) and 7 isolates (20%), respectively. Two aEPEC isolates each were found for O145:H28, ONT:H11, ONT:H8, ONT:H2, while three aEPEC isolates were untypeable for both O- and H-type.

### 3.3. Virulence profile

Virulence gene profiling was performed on all isolates. For 251 of all 282 *stx*<sup>+</sup> isolates the *eae* gene was detected (=AE-STECS), within the most common STEC serotypes (O157:H7, O26:H11, O103:H2, O145:H28 and O111:H8), only one isolate *E. coli* O157:H7 and one isolate *E. coli* O145:H28 were *eae*-negative.

The NLE genes harbored by O1-122 and O1-71 were detected in most of the AE-STECS isolates, including the most common STEC serotypes (Table V-1). The most common NLE genes in AE-STECS isolated from food were *ent/espL2* (68%) and *nleH1-2* (68%). In accordance with Franz *et al.* (2015) the NLE genes were significantly less common in the STEC isolates in comparison to the AE-STECS ( $P < 0.05$ ). In addition, the NLE genes were significantly less common in the aEPEC isolates compared to the AE-STECS ( $P < 0.05$ ) (Table V-2). The apathogenic *E. coli* showed a clear absence of NLE genes, except for one strain with *nleE*. However, significantly more NLE genes were detected in AE-STECS human isolates compared to the AE-STECS food isolates ( $P < 0.05$ ), except for *nleF* (Table V-3). Other virulence factors encoded on plasmids such as *ehxA* and *katP* were significantly more common in AE-STECS compared to STEC ( $P > 0.05$ ) (Table V-2). The most frequently detected gene was *ehxA*. None of the plasmid encoded virulence factors appeared to be more prevalent in human AE-STECS isolates compared to food AE-STECS isolates ( $P < 0.05$ ) (Table V-3). STEC autoagglutinating adhesin (*saal*) was detected in only one AE-STECS isolate (*E. coli* O157:H7), two STEC isolates (O55:HNT, ONT:HNT) and one aEPEC isolates (ONT:HNT). Subtilase-like serine protease cytotoxin gene (*subA*) was found in none of the tested isolates. Again the apathogenic *E. coli* isolates showed a clear absence of the plasmid encoded virulence genes.



Finally, the presence of *terB* was investigated as marker for the gene complex essential for the resistance to the strong oxidizing agent tellurite. This gene was identified in most AE-STECS and STEC isolates, except in AE-STECS O103:H2 of which only 36% were positive. However, no significant difference in prevalence was found between the AE-STECS and STEC isolates ( $P > 0.05$ ). On the other hand, *terB* appeared to be more prevalent in human AE-STECS isolates compared to food AE-STECS isolates ( $P < 0.05$ ). Few apathogenic *E. coli* isolates (18%) possessed this gene. On the other hand significantly more aEPEC strains possessed this resistance gene compared to both STEC and the apathogenic *E. coli* ( $P < 0.05$ ).

**Table V-2. Summary of the virulence genes and the different pathotypes based on the odds ratio (OR) for all food isolates belonging to *eae*-positive STEC (AE-STECS) (n=212), *eae*-negative STEC (STEC) (n=30), enteropathogenic *E. coli* (EPEC) (n=35) and by this study termed apathogenic *E. coli* (EC) (n=11).**

	Target	AE-STECS vs. STEC	AE-STECS vs. EPEC	AE-STECS vs. EC	STEC vs. EPEC	STEC vs. EC	EPEC vs. EC
Plasmid	<i>ehxA</i>	6.07****		27.3***			13*
	<i>saa</i>						
	<i>subA</i>						
	<i>katP</i>	18.48****	2.93*	25.31***	0.16**		
O122	<i>ent/espL2</i>	19.48****			0.02****	<sup>a</sup> 1.11****	
	<i>nleE</i>	15.15****		16.84*	0.04****		25*
O71	<i>nleA</i>	32.48**			0.02**	<sup>a</sup> 0.34**	
	<i>nleH1-2</i>	19.06****			0.04****	<sup>a</sup> 1.11****	
	<i>nleF</i>	20.11***			0.03***	<sup>a</sup> 0.71***	
	<i>terB</i>			13.85**	0.10*		74.25****

Bonferroni adjusted p-values; \*\*\*\*  $P \leq 0.0001$ , \*\*\*  $P \leq 0.001$ , \*\*  $P \leq 0.01$ , \*  $P \leq 0.05$ , empty cells mean no significant association.<sup>a</sup> One quadrant of the 2x2 contingency table contains a zero. For calculation of the odds-ratio the zero is replaced by 1. ORs indicate whether a virulence gene is more (OR>1) or less (OR<1) likely to be associated with isolates belonging to a certain group of *E. coli* compared to another group of *E. coli*.

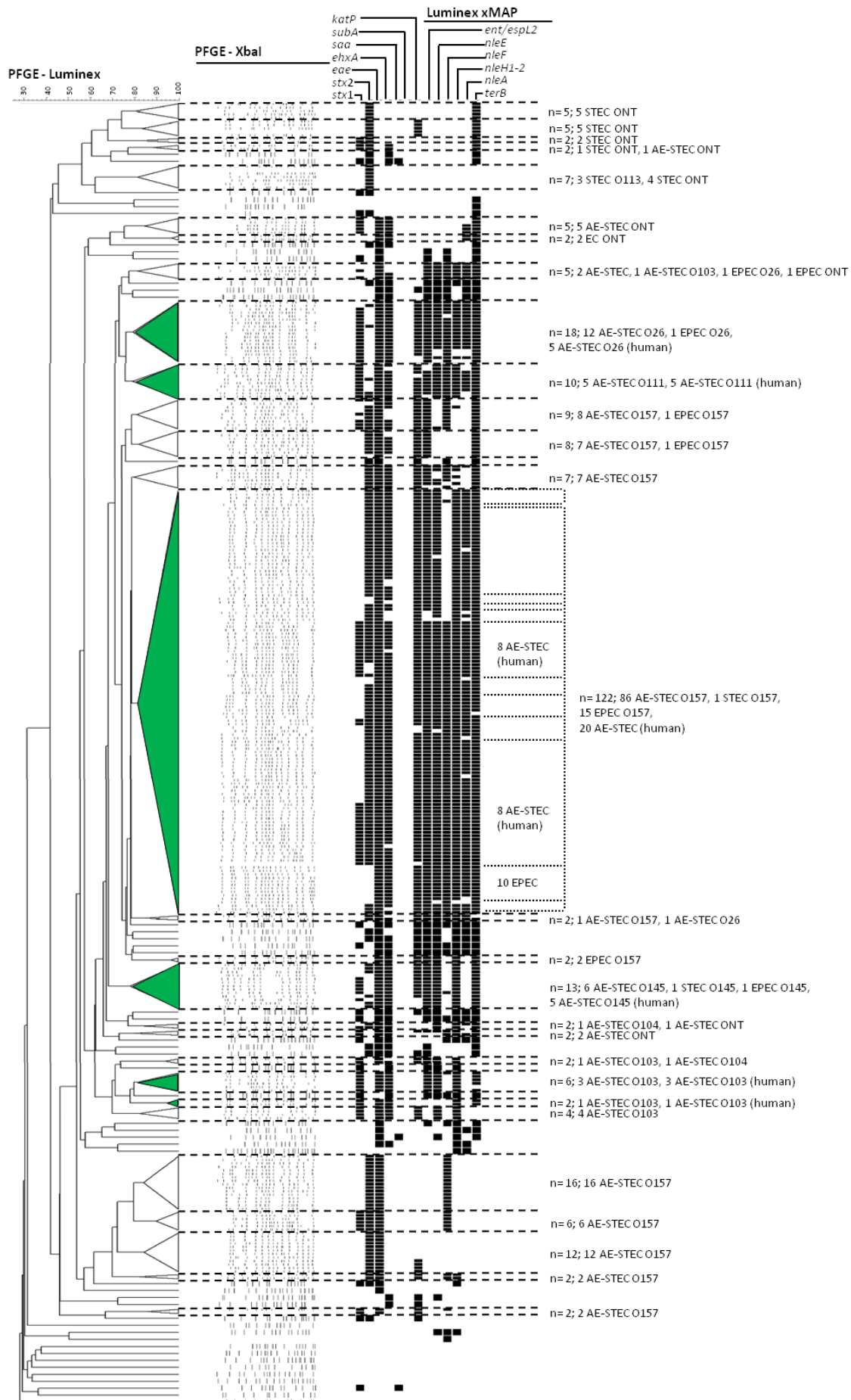
Table V-3. Summary of the virulence genes and the origin (food, n= 212; human, n=39) of the AE-STE $C$  isolates based on the odds ratio (OR).

	Target	AE-STE $C$ (human) vs. AE-STE $C$ (food)	
		Odds ratio	P-value
Plasmid	<i>ehxA</i>		
	<i>saa</i>		
	<i>subA</i>		
	<i>katP</i>		
O122	<i>ent/espL2</i>	17.6	0.005
	<i>nleE</i>	22.6	0.007
O171	<i>nleA</i>	3	0.007
	<i>nleH1-2</i>	17.9	0.005
	<i>nleF</i>		
	<i>terB</i>	12.4	0.014

### 3.4. PFGE

The combined analysis of both PFGE and virulence profile resulted overall in 28 pulsogroups (Figure V-1). Eleven of these pulsogroups contained isolates belonging to STEC serogroup O157 (AE-STE $C$ , STEC, aEPEC). The largest of these O157 pulsogroups (122 isolates) contained all 20 *E. coli* O157 isolates from humans in addition to the single *eae*-negative STEC O157:H7 isolates and 15 of the 19 aEPEC O157:H7 isolates. Twelve pulsosubgroups were found within this pulsogroup, most of the human isolates were clustered in two pulsosubgroups and ten of the aEPEC isolates were clustered in one exclusive pulsosubgroup. Most *E. coli* O26 isolates were clustered in three pulsogroups, while most of the *E. coli* O111 and O145 isolates were clustered in one pulsogroup each. Each time the human isolates of these three serogroups were clustered in one serogroup specific pulsogroup. Remarkably, most of the *E. coli* O103 isolates were divided over four pulsogroups and the human isolates were not limited to one pulsogroup. Furthermore, the one human *eae*-negative (O103) STEC isolate was a single isolate. Most of the isolates with untypable O- and/or H-type clustered in 6 pulsogroups. One of these pulsogroups contained exclusively AE-STE $C$  strains and the remaining five exclusively STEC strains, except for one AE-STE $C$  isolate (ONT:H21). The remaining STEC isolates were single isolates and all apathogenic *E. coli* were single isolates, except for one small pulsogroup containing two apathogenic *E. coli* isolates.

Figure V-1. Dendrogram of combined analysis of PFGE patterns and virulence profile (*stx1*, *stx2*, *eae*, *ent/espL2*, *nleE*, *nleA*, *nleF*, *nleH1-2*, *katP*, *ehxA*, *saa*, *subA*, *terB*) of 328 *E. coli* isolates. Delineation of pulsogroups indicated by triangle was done on the basis of 80% similarity. The green triangles indicate the pulsogroups containing human isolates. Only the largest pulsogroup is subdivided in pulsosubgroup, done on the basis of 90% similarity, these are delineated by dotted lines. All isolates are from food origin, except for 40 human isolates, which are indicated as (human). n= number of isolates and description of strain characteristics in pulsogroup.



#### 4. Discussion

In the past decade, besides the *E. coli* O157:H7 strains, many non-O157:H7 STEC serotypes, such as O26:H11, O103:H2, O111:H8, O145:H28, were linked to severe human illness. However, the pathogenicity of a single strain remains difficult to predict (Bugarel *et al.*, 2010). Although, the *stx1* and/or *stx2* presence represent the main STEC virulence factors, it is not suitable enough for the identification of STEC strains with high potential to cause disease in humans (Coombes *et al.*, 2008). In the present study we attempted the combined approach of PFGE fingerprinting, virulence profiling and serotyping to group *E. coli* strains of mainly food origin and supplemented with recent isolated human strains in Belgium.

Five seropathotypes (A through E) were proposed by Karmali *et al.* (2003) based on the reported frequency in human illness and association with outbreaks of certain STEC serotypes. For the present study, of the 212 AE-STEC isolates from food the majority (89%) can be classified into the two most pathogenic seropathotypes A and B. On the other hand of the 30 STEC isolates from food only a few (7%) could be classified into these two seropathotypes. According to this system the remainder of the isolates (n= 46) are of low virulence potential. However, Franz *et al.* (2015) noted that this classification system is purely based on the reported STEC cases and outbreaks in the past, while these databases were large, they were still limited. A number of surveillance and epidemiological studies have used phage typing to associate phage type and strain virulence. (Cowley *et al.*, 2015; Mora *et al.*, 2004)

The detection of plasmid and PAI encoded virulence factors appeared to be a useful marker in the virulence profile of the individual isolates. While the well-known LEE PAI consist of very conserved core regions, OI-122 contains various mobile genetic elements and is therefore less stable. For this reason, the detection of only the flanking sequences of the PAI might give an incorrect view of its mobile genetic elements. Some of the most characteristic NLE genes were included for OI-122 (*ent/espL2*, *nleB*) and OI-71 (*nleH1-2*, *nleA*, *nleF*). Nevertheless, the exact function of these genes in the pathogenesis of STEC is still largely unknown (Bugarel *et al.*, 2011; Karmali *et al.*, 2003). In the present study, the NLE genes of both OI-122 and OI-71 were most prevalent in the AE-STEC and aEPEC isolates, Morabito *et al.* (2003) already reported a strong correlation between the LEE and OI-122 presence. Even a higher prevalence of NLE genes was detected in the human AE-STEC isolates, it must be recognized that the number of human isolates in this study was rather limited in comparison to the food isolates. Buvens *et al.* (2012) examined 265 human isolates from Belgium and noted a strong correlation between OI-122 and STEC strains associated with severe disease. Bugarel *et al.* (2011) reported the same correlation between NLE genes and EHEC strains frequently involved in outbreaks. On the other hand, a less

significant correlation was observed regarding the plasmid-encoded virulence factors (Bugarel *et al.*, 2010). In the present study, these factors showed similar prevalences as the NLE genes, except for *saa* and *subA*. The latter two genes are both encoded by the plasmid pO113 mainly found in LEE-negative STEC strains. However, no *subA* and very low *saa* presence was detected in both AE-STEC and STEC in our study, in contrast with other studies where higher prevalences were observed (Bai *et al.*, 2015; Franz *et al.*, 2015; Tozzoli *et al.*, 2010). However, the method applied to detection and isolation the isolates used in this study were dependent on the laboratories performing the analyses. Moreover, only a random selection of all these collected isolates was included in the study. Therefore, it should be noted that the resulting collection may do not fully represent the natural STEC population found in food. Moreover, it impedes the comparison between culture collection and studies. The virulence profile of the isolates indicate, in accordance with the seropathotype scheme, that most of the AE-STEC isolates appear to possess a higher virulence potential compared to the STEC isolates. However, no single virulence factor tested seems to be specific enough to completely distinguish between virulence potentials. Moreover, not only the pathogen factors influences the acquisition of STEC-associated disease in humans. Several host-related factors are equally important, such as age, immunity and use of antibiotics (Rivas *et al.*, 2014; Verstraete *et al.*, 2013).

Using the PFGE-based genomic fingerprint and the virulence profile to group similar isolates together, several pulsogroups were created. The most common STEC serogroups clustered in one or more pulsogroups. The Belgian human isolates, randomly selected over time between 2011 and 2013, clustered per serogroup in one pulsogroup, except for the O103 strains. This might indicate that the food isolates belonging to those pulsogroups have a higher virulence potential. This assumption should be further investigated using more human isolates and virulence assays. However, the potential virulent pulsogroups of *E. coli* O157, O26 and O145 isolates also contained aEPEC isolates with a high degree of similarity with the AE-STEC isolates. Previous studies have already reported closely related EHEC and aEPEC isolates, the latter only lacking *stx*-converting bacteriophage. Therefore, it can be assumed that many of these aEPEC isolates were derived from EHEC isolates by losing their *stx*-genes (Bugarel *et al.*, 2011; Ferdous *et al.*, 2015; Haugum *et al.*, 2014; Joris *et al.*, 2011). While routine screening methods are primarily based upon the presence of *stx* genes, these potential virulent *stx*-negative aEPEC isolates might also pose a public health risk (Ferdous *et al.*, 2015). Therefore, the classical view of STEC as one of the most important diarrheagenic *E. coli* pathotypes could be brought into question, because the virulence backbone of the *E. coli* strains is clearly of major importance since this backbone may prove the difference between low-risk and high-risk STEC strains after integration by the *stx*-converting bacteriophage. By characterizing *E. coli* isolates as described in this study, potential

virulent but *stx* negative isolates might still be recognized. However, since the STEC detection in food is *stx*-based, these potential virulent strains will not be isolated.

Due to increased awareness and improved detection methods an increasing number of STEC strains are being detected in human, food, animal and environmental sources. These methods are often based on enrichment and /or isolation media supplemented with tellurite, while for other *E. coli* this supplement is rarely used. This can partly explain the significantly higher prevalence of the tellurite resistance gene *terB* gene in STEC and AE-STEC compared to the apathogenic *E. coli*. It should be noted that the term “apathogenic” *E. coli* in this study denotes those *E. coli* isolates negative for all tested pathotype specific genes. Therefore, these isolates may still harbor virulence genes not included in this study.

In summary, we have characterized a collection of isolates of *E. coli* O157, O26, O103, O111, O145 and others originating from food in Belgium between 2000 and 2014. This characterization revealed pulsogroups based on PGFE patterns and virulence profiles. Certain pulsogroups contain isolates with a higher virulence potential and pose therefore a potential public health risk. When in the future strains are isolated from food, their virulence potential could be estimated using the described characterization method and pulsogroups in this study.

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## **Chapter 6: Comparison of Droplet Digital PCR and qPCR for the Quantification of Shiga Toxin-Producing *Escherichia coli* in Bovine Feces**

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Adapted from:

Verhaegen B., De Reu K, De Zutter L., Verstraete K., Heyndrickx M., Van Coillie E.. (2016)  
Comparison of Droplet Digital PCR and qPCR for the Quantification of Shiga Toxin-Producing  
*Escherichia coli* in Bovine Feces. Toxins. 8, 157, 1-11.

**Abstract**

Cattle are considered to be the main reservoir for Shiga toxin-producing *Escherichia coli* (STEC) and are often the direct or indirect source of STEC outbreaks in humans. Accurate measurement of the concentration of shed STEC in cattle feces could be a key answer to questions concerning transmission of STEC, contamination sources and efficiency of treatments at farm level. Infected animals can be identified and the contamination level quantified by real-time quantitative PCR (qPCR), which has its specific limitations. Droplet digital PCR (ddPCR) has been proposed as a method to overcome many of the drawbacks of qPCR. This end-point amplification PCR is capable of absolute quantification independent from any reference material and is less prone to PCR inhibition than qPCR. In this study, the qPCR-based protocol described by Verstraete *et al.* (2014) for Shiga toxin genes *stx1* and *stx2* and the intimin gene *eae* quantification was optimized for ddPCR analysis. The properties of ddPCR and qPCR using two different mastermixes (EMM: TaqMan® Environmental Master Mix 2.0; UMM: TaqMan® Universal PCR Master Mix) were evaluated, using standard curves and both artificial and natural contaminated cattle fecal samples. In addition, the susceptibility of these assays to PCR-inhibitors was investigated. Evaluation of the standard curves and both artificial and natural contaminated cattle fecal samples suggested a very good agreement between qPCR using EMM and ddPCR. Furthermore, similar sensitivities and no PCR inhibition were recorded for both assays. On the other hand, qPCR using UMM was clearly prone to PCR inhibition. In conclusion, the ddPCR technique shows potential for the accurate absolute quantification of STEC on the farms, without relying on standardized reference material.



## 1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxin-producing *E. coli* (VTEC), remains a major foodborne pathogen of worldwide concern. STEC can be transmitted to humans through many different routes, either by direct contact with STEC contaminated fecal material, or indirectly via consumption of fecally contaminated meat, milk, fruits, vegetables or water (Jinneman *et al.*, 2012; Nataro and Kaper, 1998). Ruminants, especially cattle, are colonized by STEC and regarded as the natural reservoir (Nataro and Kaper, 1998). STEC can be pathogenic to humans, causing mild to severe clinical symptoms (Lorenz *et al.*, 2013). *E. coli* O157:H7 remains the serotype which have been most frequently associated with severe symptoms, therefore most studies have examined the epidemiology of *E. coli* O157:H7 in cattle populations. However, the non-O157 STEC serogroups, such as O26, O103, O111 and O145, are increasingly being recognized and reported as important foodborne pathogens. Still, much less is known about these STEC serogroups (EFSA, 2016; Thomas *et al.*, 2012). The shedding pattern of STEC in cattle is mostly low in level, but can vary from 10 to 10<sup>9</sup> CFU per gram feces, and is mostly short in duration (Munns *et al.*, 2015). However, some animals may be more persistent carriers of the pathogen or shed at higher levels (at least 10<sup>4</sup> CFU per gram feces) for a longer period (>10 days) than others. These so-called “super-shedders” have a major impact on the on-farm prevalence and transmission, as well as in food contaminations (Duffy *et al.*, 2014; Matthews *et al.*, 2006). The detection of these super-shedders is often performed using culture-based techniques to enumerate STEC in feces, such as direct plating, spiral plating and the most probable number (MPN) technique. These approaches ensure quantification of > 10<sup>2</sup> CFU/g feces, however the stressed and injured cells will not be counted (Lawal *et al.*, 2015). Furthermore, the lack of an efficient selective isolation medium for all STEC strains makes these culture techniques too labor intensive to process large numbers of samples and even ineffective for various STEC strains (Verhaegen *et al.*, 2015a (e. i. chapter 3)). A culture-independent method, such as quantitative polymerase chain reaction (qPCR), is often applied to quantify STEC in feces (Verstraete *et al.*, 2014). However, this method requires a DNA extraction and the limit of quantification is higher (10<sup>3</sup> – 10<sup>4</sup> CFU/g) compared to the culture-dependent techniques (Ahmed *et al.*, 2015; Lawal *et al.*, 2015; Luedtke *et al.*, 2014; Munns *et al.*, 2015; Noll *et al.*, 2015). Furthermore, this approach is based on relative quantification and totally dependent on the accuracy of the standard curve construction (Bustin and Nolan, 2004). Recently, a “third-generation PCR” or droplet digital PCR (ddPCR) has been developed. This technique allows for absolute quantification of target DNA molecules without the requirement for a standard curve. The technique is based on partitioning of the PCR sample into many thousands of droplets so that each contains one (or a few) or no copies of the target DNA. The absolute number of target DNA in the sample is calculated directly from the ratio of

the positive to the total of partitions using binomial Poisson statistics (Baker, 2012). The PCR amplification occurs in each droplet. The fluorescence signal of each droplet is individually counted. Since ddPCR is an end-point PCR, it is suggested to be more flexible concerning sample quality and thus less prone to PCR inhibition (Hindson *et al.*, 2013; Pinheiro *et al.*, 2012).

In this study, we optimized a qPCR protocol for the quantification of the main virulence genes of STEC for ddPCR use. Furthermore, we compared the sensitivity and resistance to PCR inhibition of both qPCR and ddPCR assays, using artificially and naturally contaminated cattle feces. Alternatively, this optimized protocol for the ddPCR could be used for the detection of STEC in food.

## 2. *Materials and Methods*

### 2.1. *Strains*

Bacterial strains MB3936 (STEC O26; *stx1+* *stx2+* *eae+*) and MB4378 (STEC O138; *stx2e+*) were used in this study, both were isolated from humans. Both strains carry single copies of the tested genes. Both strains were stored at -80 °C using Pro-Lab Microbank cryovials (Pro-Lab, Richmond Hill, ON, Canada) according to the manufacturer's instructions. Strains were cultured on Tryptone Soy Agar (TSA; Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37 °C for 24 h. A single colony from these culture plates was transferred into Tryptone Soy Broth (TSB; Oxoid). After incubation at 37°C for 24h the genomic DNA (gDNA) was purified using DNeasy Blood & Tissue kit (Qiagen Inc, Valencia, CA, US) according to the manufacturer's instruction for Gram-negative bacteria with an additional RNase step, and eluted in a final volume of 200 µl elution buffer. The concentration of the gDNA was measured using a Quantus™ fluorometer (Promega, Madison, WI, US). The following formula was used for calculation of the mass (M) of one genome:

$$M = n \times 1.096 \times 10^{-21} \frac{\text{gram}}{\text{bp}}$$

For *E. coli* strain O157:H7 EDL933, the genome length (n) was determined as  $5.53 \times 10^6$  bp (Perna *et al.*, 2001).

Both gDNA preparations were diluted in nuclease-free water (Qiagen) to  $10^6$  copies/µL and stored as stock template at -20°C until use.

## 2.2. qPCR assays

qPCR for the quantification of the STEC virulence genes as described by Verstraete *et al.* (2014) was used. In this assay, four different primer sets are used in singleplex, one primer set for the quantification of the subtypes of *stx1*, one for the quantification of *eae* and two primer sets for the quantification of the subtypes of *stx2*. Two master mix types were used, namely TaqMan® Environmental Master Mix 2.0 (EMM; Life Technologies, Carlsbad, CA, USA) and TaqMan® Universal PCR Master Mix (UMM; Life Technologies). Each qPCR mixture (25 µl including 5 µl DNA template) contained: 1x PCR master mix (EMM or UMM), 300 nM of both F/R primers of the respective primer set and 100 nM of 5'-FAM labeled probe (Eurogentec, Seraing, Belgium) (Table VI-1). The thermal protocol was as follows: initial incubation at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 1 min annealing and elongation at 60 °C, and cooling at 40 °C for 30 s. For the use of UMM, which contains Uracil-DNA Glycosylase (UNG), an initial enzyme activation step was included at 50°C for 2 min. A standard curve of a serial dilution of gDNA of STEC strain MB3936 for *eae*, *stx1* and *stx2* (set a, specific for *stx2a*, *stx2b*, *stx2c*, *stx2g*) and of MB4378 for *stx2* (set b, specific for *stx2d* and *stx2e*) was utilized. All qPCR assays were performed on a LightCycler® 480 (Roche Diagnostics, Vilvoorde, Belgium) using the LightCycler 480 Software version 1.5.0 (Roche Diagnostics).

Table VI-1. Overview of primers/probes sequences (all designed by (Verstraete *et al.*, 2014)) and labels used in this study

Gene	Primer or probe*	Sequence (5'-3')	Labeling (5'-3')	
			qPCR	ddPCR
<i>eae</i>	<i>eae</i> -F	GGA AGC CAA AGC GCA CAA	-	-
	<i>eae</i> -R	GGC ICG AGC IGT CAC TTT ATA A	-	-
	<i>eae</i> -P	TAC CAG GCT ATT TTG CCI GCT TAT GTG C	FAM-BHQ-1	FAM-ZEN-IBFQ
<i>stx1</i>	<i>stx1</i> -F	GAC GCA GTC TGT IGC AAG AG	-	-
	<i>stx1</i> -R	CGA AAA CGI AAA GCT TCA GCT G	-	-
	<i>stx1</i> -P	ATG TTA CGG TTT GTT ACT GTG	FAM-MGBNFQ	FAM-ZEN-IBFQ
<i>stx2</i>	<i>stx2</i> -F	TCA GGC AIA TAC AGA GAG AAT TTC G	-	-
	<i>stx2</i> -R (set a)	CCG GIG TCA TCG TAT ACA CAG	-	-
	<i>stx2</i> -R (set b)	CCG GIG TCA TCG TAT AAA CAG	-	-
	<i>stx2</i> -P	CAC TGT CTG AAA CTG CT	FAM-MGBNFQ	FAM-ZEN-IBFQ
IAC plasmid	<i>stx1</i> -F	GAC GCA GTC TGT IGC AAG AG	-	-
	<i>stx1</i> -R	CGA AAA CGI AAA GCT TCA GCT G	-	-
	<i>eae</i> -P	TAC CAG GCT ATT TTG CCI GCT TAT GTG C	HEX-BHQ-1	HEX-ZEN-IBFQ

\*Forward primers with suffix -F; Reverse primers with suffix -R; Probes with suffix -P.

MGBNFB: minor groove-binding non-fluorescent quencher (Applied Biosystems), BHQ-1: black hole quencher (Eurogentec), ZEN-IBFQ: internal ZEN™ fluorescence quencher- Iowa Black fluorescence quencher (IDT)

### 2.3. *ddPCR assays*

Before the comparison of quantification of the STEC virulence genes using qPCR and ddPCR, the ddPCR assays were first optimized in two steps: optimization of 1) the thermal protocol and 2) the concentration and labeling of the primers and probes. For the optimization of the thermal protocol a range of annealing temperatures (55–65 °C) were compared. For the primers and probes different concentrations and the use of regular single-quenched probes versus double-quenched ZEN probes (Integrated DNA Technologies (IDT), Coralville, IA, US) were evaluated. The ddPCR workflow and data analyses were performed according to the manufacturer's instructions. Briefly, 20 µL of each reaction mixture was loaded into a sample well of an eight-channel disposable cartridge (Bio-Rad, Marnes-la-Coquettes, France) followed by 70 µL of droplet generator oil (Bio-Rad) into the oil-wells of the cartridge. Droplets were formed in the QX200 droplet generator (Bio-Rad). Droplets were then transferred to a 96-well PCR plate, heat-sealed with foil (Bio-Rad) in a PX1™ PCR Plate Sealer (Bio-Rad), and amplified with a T100 Touch Thermal Cycler (Bio-Rad). PCR reactions were analyzed with the QX200 droplet reader (Bio-Rad) and data analysis was performed using the QuantaSoft software (Version: 1.6.6., Bio-Rad). Note that a positive control is sufficient for the ddPCR technology, instead of the standard curve for the qPCR technology.

Satisfactory separation of positive and negative droplets for the target was achieved using the following optimized reaction mixture: 10 µl of 2 x Supermix for Probe (No UTP) (Bio-Rad), 900 nM of both F/R primers, 250 nM of 5'-FAM labeled double-quenched probe in a mixture volume of 20 µl. Five µl of template DNA was added to each mixture. The optimized thermal protocol included an initial incubation step at 95°C for 5 min followed by 40 cycles of a 3-step amplification at 95°C for 15 s, 59°C for 30 s, and 60°C for 30 s, and cooling at 40 °C for 30 s. The threshold for a positive signal was set at a fluorescence amplitude of 1500. Only the reactions with more than 10,000 accepted droplets were used for analysis.

Once the conditions were optimized the dynamic range of both qPCR and ddPCR assay were compared. For this purpose, each stock template was serially diluted from  $2 \times 10^5$  to  $2 \times 10^0$  target copies per µl. For each PCR assay, three replicates were performed. The degree of linearity ( $R^2$  value) and slope were calculated on the average numbers of target copies measured by qPCR and ddPCR assays.

## 2.4. Inhibition

Bile salt, a known PCR inhibitor present in faeces (Thompson *et al.*, 2014) was selected to evaluate the effect of PCR inhibition using qPCR with EMM (qPCR-EMM) and UMM (qPCR-UMM) and ddPCR. A concentration range (0, 0.125, 0.25, 0.375, 0.5, 1 µg Ox Bile Extract (Oxoid) per µl reaction mixture) was tested. This range of concentrations was tested based on a previous study which showed a change in signal output of the qPCR (Thompson *et al.*, 2014). To each reaction mixture  $10^3$  target copies were added. For each PCR assay, three replicates were performed.

## 2.5. Internal amplification control (IAC)

As internal amplification control a synthetic gene sequence inserted in a plasmid cloning vector synthesized by Integrated DNA Technologies (IDT) was used. The synthetic gene (5'- GAC GCA GTC TGT TGC AAG AG -TATATA- TAC CAG GCT ATT TTG CCT GCT TAT GTG C -TATATA- C AGC TGA AGC TTT ACG TTT TCG -3') contained the primer/probe binding sites (underlined) for the *stx1*-forward-primer, the *eae*-probe and the *stx1*-reverse-primer. The concentration was measured using a Quantus™ fluorometer (Promega), diluted in nuclease-free water to  $10^2$  copies/µl and stored as stock IAC at -20°C until use.

Five microliters of the stock IAC was added to each *stx1* reaction. Additionally, 100 nM and 250 nM 5'-HEX labeled *eae*-probe for qPCR and ddPCR, respectively, was added for the detection of the IAC. The IAC at a concentration of  $10^2$  copies/µl had no influence on the *stx1* quantification. The comparison of the obtained results with the results of a parallel *stx1* reaction containing the same amount of IAC, but in the absence of sample DNA enables the determination of the level of PCR inhibition. The IAC was included in all assays for the analyses of artificial and natural contaminated fecal samples.

## 2.6. Artificial STEC contaminated fecal samples

Multiple cattle fecal samples were taken from a local combined (beef and dairy) farm. Eight samples that were found negative for the *stx* genes by qPCR assay, were used for artificial contamination. These fecal samples were subdivided in subsamples of 0.25 gram feces. Subsamples were inoculated with the appropriate volumes of diluted fresh overnight cultures of MB3936 or MB4378, to obtain 24 inoculation levels ranging between  $1,2 \times 10^2$  and  $1,1 \times 10^7$  CFU per gram feces. The artificially contaminated fecal samples (0.25 gram) were subjected to DNA extraction using the QIAmp DNA stool Mini kit (Qiagen). The extracted DNA was then analyzed in duplicate by both qPCR, using EMM and UMM, and ddPCR assays. The gene copy numbers in 1 g artificial contaminated feces were calculated while accounting for the dilution factor in the PCR assay ( $\times 160$ ). The limit of quantification (LOQ) was defined as the lowest number of organisms

that can be quantified (Verstraete *et al.*, 2014). The viable cell counts of the diluted cultures for artificial inoculation were determined by plating in triplicate onto Tryptone Soy Agar (TSA) using a spiral plater (Eddy Jet Spiral Plater, IUL instruments, Barcelona, Spain). After incubation of the plates at 37°C for 24 h colonies were enumerated.

### **2.7. Natural STEC contaminated fecal samples**

Samples from ten animals were taken at a culture-confirmed STEC-positive local farm. From each animal two samples were collected. First, a recto-anal-mucosal swab (RAMS) was taken, using a sterile floqswab (Copan Diagnostics Inc., Murrieta, CA, USA), followed by a fecal sample taken directly from the rectum. Both samples were placed in plastic bags and transported on ice packs. Upon arrival in the laboratory 0.25 gram of all samples were subjected to DNA extraction using the QIAmp DNA stool Mini kit (Qiagen) and analyzed by both qPCR, using EMM and UMM, and ddPCR assays, as described above.

### **2.8. Statistical analysis**

All measured numbers of genomic copies were log-transformed prior to analyses. Significant differences between gene copy numbers measured by qPCR-EMM, qPCR-UMM and ddPCR assays were determined using the standard paired t tests. The significance level of all analyses was set at 0.05. The statistical analyses were performed with the software R (<http://www.R-project.org>).

## **3. Results**

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### **3.1. Comparison of qPCR and ddPCR standard curves**

Diluted series of gDNA of strains MB3936 and MB4378 were analyzed in order to compare qPCR using TaqMan® Environmental Master Mix 2.0 (EMM) and ddPCR performance. Both assays exhibited an excellent degree of linearity ( $R^2$ : 0.9959 to 0.9999). The slope values in qPCR ranged from -3.25 to -3.31, equivalent to 101% to 103% PCR efficiency. In ddPCR, the PCR efficiency ranged from 96% to 105%. Both assays were able to quantify the lowest tested gDNA concentration ( $2 \times 10^0$  target copies per  $\mu$ l). However, for ddPCR reaction saturation, or state in which every droplet contained at least one target copy, was reached at a concentration of  $2 \times 10^5$  target copies/ $\mu$ l and therefore it was impossible to quantify this high concentration (Table VI-2).

The correlation between the qPCR-EMM and ddPCR measurements showed a degree of linearity ( $R^2$ : 0.9899 to 0.9998) very close to 1. This suggests a very good correlation between qPCR and ddPCR assays.

Table VI-2. ddPCR reaction saturation percentages of the standard curves for *eae*, *stx1*, *stx2* (set a) and *stx2* (set b) quantification.

Concentration (copies per $\mu$ l template)	Gene			
	<i>eae</i>	<i>stx1</i>	<i>stx2</i> (set a)	<i>stx2</i> (set b)
$2 \times 10^5$	100%	100%	100%	100%
$2 \times 10^4$	87%	87%	87%	88%
$2 \times 10^3$	19%	19%	19%	21%
$2 \times 10^2$	2.03%	1.96%	2.06%	2.11%
$2 \times 10^1$	0.22%	0.26%	0.20%	0.18%
$10^1$	0.12%	0.13%	0.13%	0.17%

### 3.2. Inhibition

Figure VI-1 shows the effect of an increasing amount of bile salts (0, 0.125, 0.25, 0.375, 0.5, 1  $\mu$ g per  $\mu$ l reaction mixture) on qPCR-EMM, qPCR using TaqMan® Universal PCR Master Mix (UMM) and ddPCR. Concentration of bile salts up to 0.5  $\mu$ g/ $\mu$ l in the PCR mixture did not affect the qPCR-EMM and ddPCR results. In contrast to qPCR-EMM, with ddPCR no measurements were possible with 1  $\mu$ g/ $\mu$ l bile salts, because at this high concentration it was not possible to generate droplets. For qPCR-UMM, the amplification efficiency was substantially inhibited by increasing the concentration of bile salts. The PCR assays for *eae* and *stx2* (set b) seemed to be more prone to PCR inhibition by bile salts in comparison to *stx2* (set a) and *stx1*.

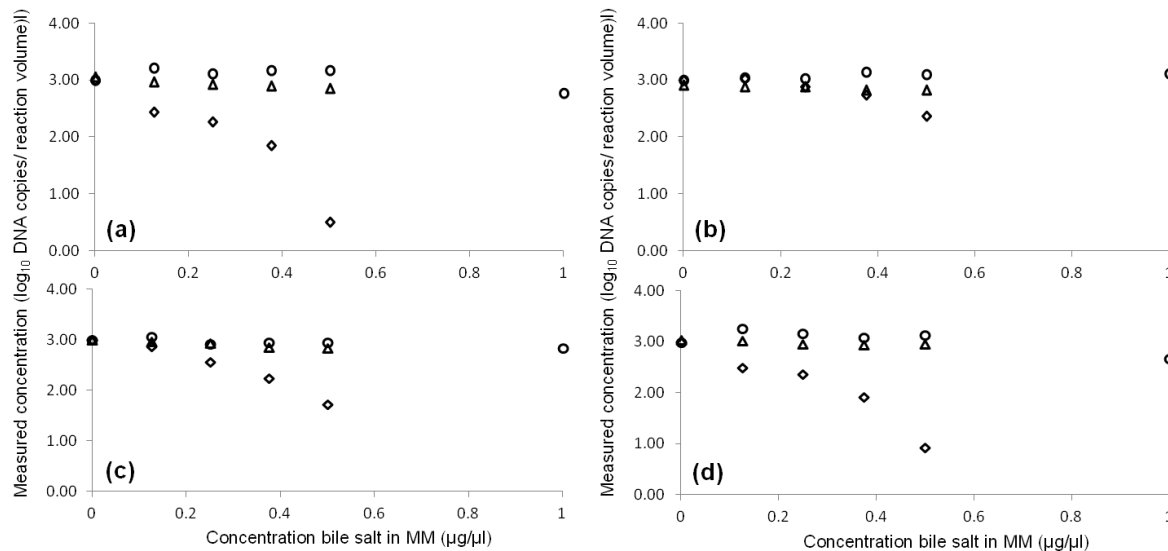
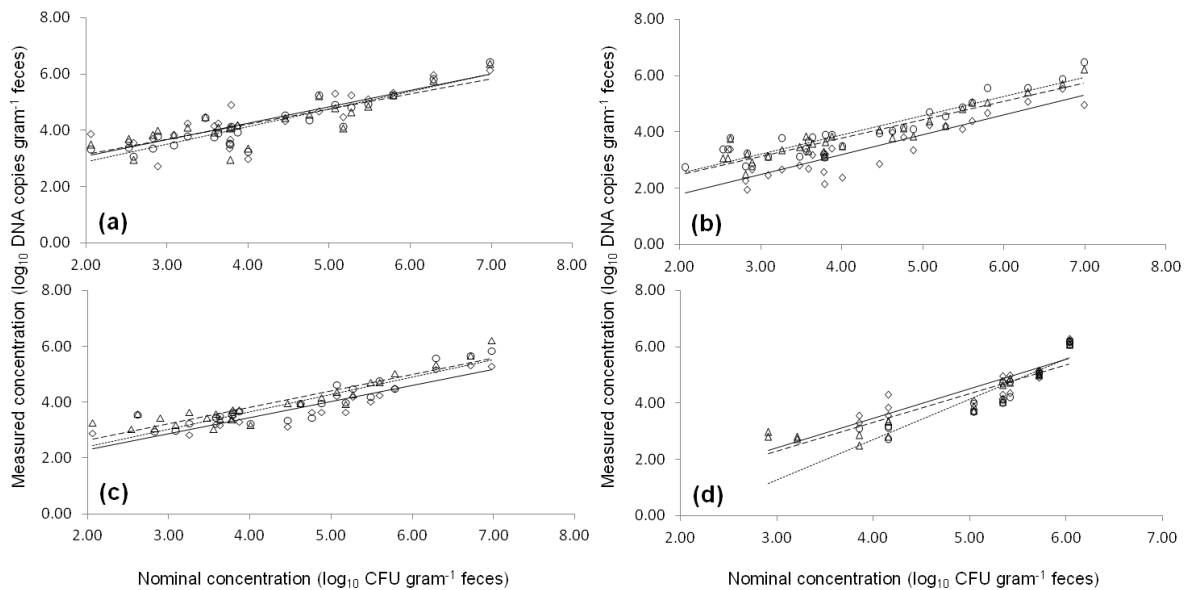


Figure VI-1. Influence of increasing concentrations of bile salt on the measured target DNA copies in qPCR-EMM (○), qPCR-UMM (◊) and ddPCR (△) for *eae* (a), *stx1* (b), *stx2* (set a) (c) and *stx2* (set b) (d) quantification. MM: mastermix

### 3.3. Artificial contaminated fecal sample

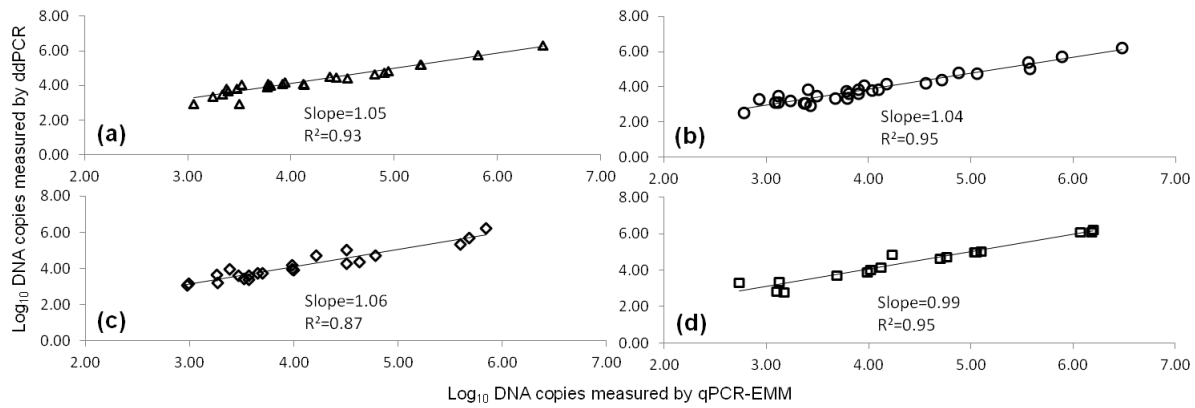
Two STEC strains were separately added to *stx*- and *eae*-negative cattle feces at different concentrations, and inoculated samples were subjected to qPCR-EMM, qPCR-UMM and ddPCR analysis (Figure VI-2). The limit of quantification for both qPCR-EMM and ddPCR was 2.75 log copies g<sup>-1</sup> feces for *stx1* and *stx2* (set b) and 3.06 log copies g<sup>-1</sup> feces for *eae* and *stx2* (set a). The results of the qPCR-UMM were generally lower compared to qPCR-EMM and ddPCR ( $P < 0.05$ ), except for the *eae* assays and *stx2* (set b) (Figure VI-2). However, the results of the Internal Amplification Control (IAC) showed no PCR inhibition in any of the samples.



**Figure VI-2** Quantification of *eae* (a), *stx1* (b), *stx2* (set a) (c) and *stx2* (set b) (d) by qPCR-EMM (○,.....), qPCR-UMM (◇,——) and ddPCR (△,— —) in cattle fecal samples artificially inoculated with STEC cells. Artificial inoculation was performed using various contamination levels of strain MB3936 (*stx1*, *stx2* (set a) and *eae*) and MB4378 (*stx2* (set b)).

The correlation between the qPCR-EMM and ddPCR measurements (>LOQ) showed  $R^2$  values ranged from 0.87 to 0.95 depending on the target gene (Figure VI-3). No significant differences were observed for the *eae* and *stx2* (set b) assays by qPCR-EMM and ddPCR ( $P > 0.05$ ). The *stx1* qPCR-EMM measurement was significantly higher compared to ddPCR ( $P < 0.05$ ), while the *stx2* (set a) measurement was significantly lower ( $P < 0.05$ ). However, the biological importance of these findings is less meaningful.





**Figure IV-3.** The linear correlation between qPCR-EMM and ddPCR measurements for *eeae* ( $\Delta$ ,a), *stx1* ( $\circ$ ,b), *stx2* (set a) ( $\diamond$ ,c) and *stx2* (set b) ( $\square$ ,d) genes quantification in cattle fecal samples artificially inoculated with STEC cells. Artificial inoculation was performed using various contamination levels of strain MB3936 and MB4378.

### 3.4. Natural contaminated fecal samples

The number of target copies measured by qPCR-EMM, qPCR-UMM and ddPCR in fecal samples and recto-anal-mucosal swab (RAMS) of ten animals from a STEC-positive farm are presented in Figure VI-4. In all ten animals *eeae* was detected (7/10 fecal samples, 10/10 RAMS) with at least one of the three assays. *Stx1* was found in seven animals (4/7 fecal samples, 7/7 RAMS) and *stx2* in nine animals (4/9 fecal samples, 9/9 RAMS) with at least one of the three assays. In this experiment, a higher positive rate was noted from RAMS in comparison to fecal samples from the same animal. While the type of sample seemed important, no significant differences were observed between measurements by the qPCR-EMM and ddPCR assays above the limit of quantification ( $P>0.05$ ). The results of the IAC showed no PCR inhibition in any of the fecal samples or RAMS, except for fecal samples of animal 4 and 10 in the qPCR-UMM assay (Figure VI-4)

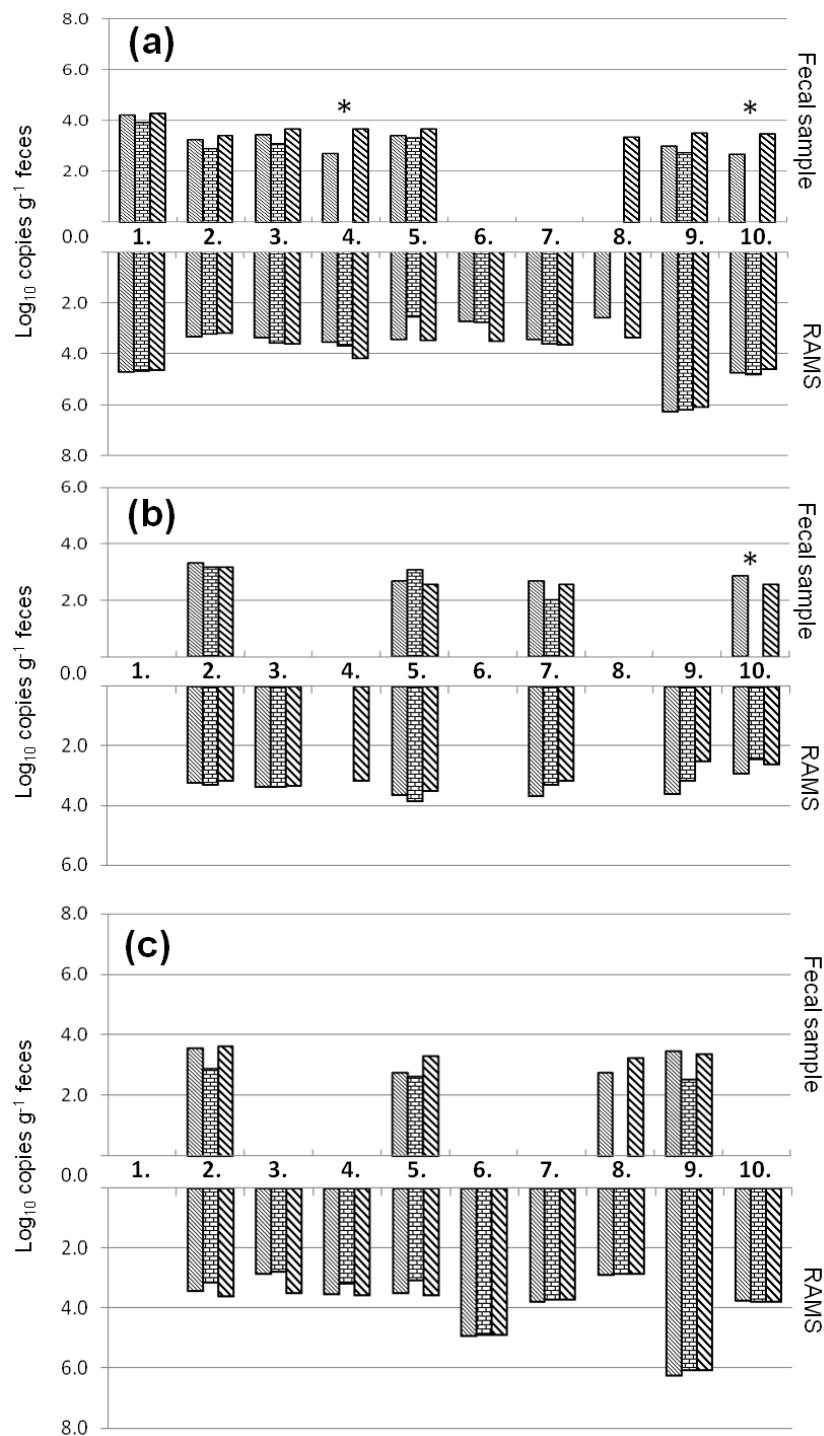


Figure VI-4. Quantification of *eae* (a), *stx1* (b) and *stx2* (c) genes by qPCR-EMM, qPCR-UMM and ddPCR in cattle fecal samples and recto-anal-mucosal swabs (RAMS) of ten animals (1-10) of a STEC-positive farm. \* Cattle fecal samples that showed PCR inhibition for the internal amplification control..

#### 4. Discussion

This study evaluated ddPCR as a technique to quantify STEC in cattle feces in comparison to conventional qPCR. After some optimization of the ddPCR assay, by using a double quenched ZEN probe, and altering the primer/probe concentrations and annealing temperatures, our results showed that ddPCR had an excellent agreement with qPCR in DNA quantification. However, since  $10^5$  target copies per  $\mu\text{l}$  template resulted in 100% saturation of positive droplets, the upper quantification limit for the ddPCR was notably lower in comparison to qPCR. Pinheiro *et al.* (2012) noted the same dynamic limit of  $10^5$  target copies per  $\mu\text{l}$  template. Therefore, the quantification of high levels of targets is a limitation of the ddPCR compared to the qPCR assay. However, in none of the ddPCR runs from naturally contaminated fecal samples in this study saturation were observed.

One of the main advantages of ddPCR compared to qPCR is that it would be less prone to inhibitors which may be present in natural samples, even after DNA purification. These inhibitors may induce a shift in the amplification curve and therefore an increase in threshold cycle (Ct) during the qPCR. Since the ddPCR is an end-point PCR, the impact of such a shift would have much less influence on the final result. In this study indeed no PCR inhibition was observed with ddPCR, neither in the presence of bile salts added, nor in the tested natural samples. However, we observed that this disadvantage of the qPCR technique can be overcome by using a qPCR mastermix specially designed for matrices with high levels of inhibitors, such as TaqMan® Environmental Master Mix 2.0 (EMM). In contrast, using TaqMan® Universal PCR Master Mix (UMM) we clearly observed PCR inhibition, but the level was shown to be assay dependent. The latter corresponds to the results of Huggett *et al.* (2008) who demonstrated that the robustness of an assay has an important impact on the susceptibility of a PCR reaction to inhibitors. Moreover, a suitable DNA purification step should prevent high levels of inhibitors, such as the bile salt concentrations used in this study.

Some studies reported an increased sensitivity of the ddPCR to detect low quantities of target DNA in comparison to qPCR (Doi *et al.*, 2015; Strain *et al.*, 2013; Sze *et al.*, 2014). Beer *et al.*, (2007) showed that low numbers of target copies in a droplet needed half of the number of cycles than the same assay conducted with a regular qPCR to reach the Ct. This reduction in required cycles could explain the higher sensitivity of the ddPCR assay. However, in the present study similar LOQs were observed for both techniques.

For the evaluation of qPCR and ddPCR using natural STEC contaminated samples both fecal and RAMS samples were investigated. More RAMS samples were found positive in comparison to the fecal samples of the same animals. Furthermore, PCR inhibition was observed in some of the

fecal samples using qPCR-UMM, while in none of the RAMS samples inhibition was detected. The benefit of using RAMS instead of fecal samples was already demonstrated by Davis *et al.* (2006) for the culture-dependent detection of STEC and has already been used for qPCR-based detection of STEC in cattle (Lawal *et al.*, 2015).

One of the main advantages of ddPCR is the accurate absolute quantification without the need to rely on a standard curve. This is an important advantage compared to qPCR because the construction of any standard curve requires accurately quantified template DNA, which might be difficult to obtain.

Despite the independence of a standard curve, the ddPCR is somewhat more time consuming and labor intensive compared to qPCR. Furthermore, the ddPCR platform is more expensive compared to qPCR. For qPCR in order to prepare, amplify and analyse 96 samples it took up to three hours, while ddPCR took up to 5.5 hours. Furthermore, the cost per reaction was remarkably higher for ddPCR (ddPCR: ~€2.80, qPCR-EMM: ~€1.60, qPCR-UMM: ~€1.20). However, the need to include (different) standard curves for the quantification of *eae*, *stx1*, *stx2* (set a) and *stx2* (set b) using qPCR somewhat mitigates the difference in cost.

Finally, the ddPCR might be able to solve a major bottleneck of the qPCR assays. While it is impossible to determine whether different genes originate from the same genome or different genomes with the qPCR, the ddPCR should in theory be able to partition an entire bacterial cell into the separate reaction chambers. These assays could provide valuable knowledge about the content of one cell. This application for the ddPCR may revolutionize STEC detection and any public health interventions.

## **5. Conclusion**

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We have demonstrated that after some optimization efforts, accurate absolute quantification of the STEC target genes was possible with ddPCR. The same sensitivity compared to qPCR was observed, while ddPCR is independent of a standard curve. The accurate measurement of the concentration of shedded STEC in feces is a key answer for questions concerning transmission of STEC, contamination sources and efficiency of treatments at farm level. Because of the low sensitivity for inhibition, this technique shows promise for microbial detection and quantification in complex samples. The ddPCR technique shows potential, not only for the detection and quantification of STEC in the farms, but also as a valuable application in food safety in general.

### **Acknowledgements**

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## **Chapter 7: General discussion**

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## 1. *Detection and isolation of STEC in food*

In the field of microbiology, **culture-based detection** remains the standard method for the detection of most pathogens, such as *Salmonella*, *Campylobacter* and *E. coli* O157. However, routine cultural detection of the non-O157 STEC strains appeared more challenging. Therefore, a number of culture-independent tests, including DNA-based and immunological assays, have been developed to aid in a rapid and reliable detection of STEC (Cronquist *et al.*, 2012). The detection of STEC consists of a serotype-based or virulence gene-based approach.

In the **serotype-based approach**, both culture-independent PCR-based methods and immunological assays are employed, in addition to the culture-based detection of *E. coli* O157. Despite the sensitivity of these methods, the number of serotypes included is limited. Therefore, an appropriate selection of STEC serotypes that should be included in the screening process is necessary. However, **geographical differences** have been reported. Whereas, some STEC serogroups are common worldwide, other serogroups appeared to be more prevalent in specific regions. The most common STEC serogroups in the European Union include O157, O26, O103, O145, O91 and O111. While serogroup O91 is less common in other countries, such as the United States, Japan and Argentina, instead O121 STEC is of more importance in the latter countries. In addition the STEC serotype O45 STEC, which is seldom isolated in EU, is of significance in the US. Moreover in Argentina, the country with highest reported incidence of HUS cases worldwide and an endemic STEC presence, the STEC serogroup O174 is frequently associated with severe disease (Brooks *et al.*, 2005; EFSA, 2016; Masana *et al.*, 2011). Besides the geographical differences, any **new emerging STEC serotypes** will not be detected using a serotype-based approach with prior selected serotypes. Moreover *stx*-negative variants, occurring in the most common STEC serotypes, lead to false positive results in terms of virulence potential. For these reasons a **virulence gene-based approach** may be more advisable.

Since the production of Stx remains the main virulence trait of STEC, various detection techniques have been described (*chapter 1*). The indirect detection of Stx by detecting Stx-encoding genes has proven to be reliable and evolved into a widely accepted screening technique to classify samples as STEC-negative or possibly STEC-positive, as recommended by the ISO/TS 13136:2012 (ISO, 2012a).

### 1.1. *Enrichment*

An enrichment step in a suitable enrichment medium is advisable prior to any detection or isolation method for STEC in food. During the enrichment step the pathogen is able to resuscitate from any **sub-lethal injury or stress** sustained during food processing and

preservation. Since the infective dose of STEC is very low, even a **low number of STEC** cells may still have a large impact on public health. Therefore after resuscitation, the pathogen should be allowed to grow to a detectable level.

In *chapter 2* non-O157 STEC strains were exposed to three types of standardized stress that may occur during food processing and preservation. These stressed STEC strains were used to evaluate the growth characteristics of five enrichment media. Since freeze stress induced the highest levels of sub-lethal injured STEC cells, the resuscitation capability of the different media were best evaluated during enrichment of freeze stressed cells. The shortest lag phase and highest maximum growth rate was observed in the non-selective medium BPW. This suggests that the presence of **selective components**, such as bile salt and brilliant green, in the other tested media are capable to inhibit the resuscitation and growth of the stressed STEC cells. Jasson *et al.* (2009) observed the same features for the enrichment of *E. coli* O157 in BPW. However, both studies were performed on pure strain cultures, the use of non-selective enrichment media is generally not recommended for the detection of a target organism in the presence of competing **background microbiota**. For this purpose cold stressed non-O157 STEC strains were inoculated in different food matrices (*chapter 4*) and enriched for 20 hours. This reduction of incubation time allows for a better workflow for laboratories and a faster detection and isolation method. After enrichment in either BPW or Brila most of the inoculated STEC strains were able to grow to a detectable level in the different **artificially contaminated food matrices**. While BPW appeared to be the superior enrichment media for stressed STEC cultures, higher levels of STEC were detected in the contaminated samples after enrichment in the Brila, as determined by real-time PCR. However, Jasson *et al.* (2009) noted a preference for BPW as the enrichment broth for *E. coli* O157 from food. They considered STEC to be capable of sufficient growth in this non-selective medium, without the risk of being overgrown by the background microbiota. In contrast, our findings suggest that this feature may be greatly dependent upon the composition of the background microbiota. Since, after enrichment of food matrices harboring high levels of background microbiota, such as sprouted soy beans, only 88% and 90% of the non-O157 STEC strains were able to grow to a detectable level in BPW and Brila, respectively. A recent study noted similar percentages of STEC strains in radish sprouts able to grow to a detectable level after enrichment in mEC (Hara-Kudo *et al.*, 2016). On the other hand, Margot *et al.* (2015) observed a lack of selectivity during enrichment of sprouted seed samples in BPW. Therefore, the **incubation temperature** for the BPW enrichment in our study was raised to 41.5°C, as suggested in multiple studies describing the isolation of STEC from sprouted seeds (Hara-Kudo *et al.*, 2000b; Kanki *et al.*, 2011; Weagant and Bound, 2001). The increased selectivity derived from this incubation temperature seemed sufficient to prevent the overgrowth of the

few STEC cells during enrichment. The use of raised incubation temperature is not a new finding, the ISO 16654:2001 for the detection of *E. coli* O157 recommends enrichment at 41.5°C. Moreover, for the enumeration of generic *E. coli* the plates are even incubated at 44°C (ISO 16649:2001), supporting the growth of *E. coli* but inhibiting the growth of coliforms. These more selective incubation conditions may be a suitable replacement for antibiotic supplements since a significant portion of the STEC strains seems sensitive for the antibiotics commonly used for the detection and isolation of STEC.

## 1.2. Isolation

After efficient sample enrichment, a real-time PCR screening technique should provide the required highly-sensitive detection of all STEC. The follow-up culture-based isolation of the pathogen is crucial in order to confirm the PCR results and further characterization of the STEC strains. The CEN ISO/TS 13136 stipulated the isolation of STEC strains from any sample that tests positive for *stx*, *eae* and serogroup-associated genes belonging to the 'big five'. After the STEC O104:H4 outbreak, this standard was amended to the ISO/TS 13136:2012 stipulating the isolation of STEC strains from any *stx*-positive sample (EFSA, 2013b). However, the pathogen isolation via selective isolation agar media can be labor-intensive and time-consuming. The hallmark of such isolation is the use of a suitable **selective and differential** culture medium. The ISO/TS 13136:2012 suggests the use of the general *E. coli* isolation medium TBX, but allows the use of any other isolation medium. Because the chance of recovery of STEC on TBX by random picking of suspect colonies is rather low, up to 50 presumptive STEC colonies should be isolated followed by colony pooling and confirmation by (q)PCR. This practice has an important impact on the labor and time requirement for cultural isolation. Therefore, a more efficient selective and/or differential isolation medium for STEC is needed. A broad variety of isolation media for STEC in general or for specific STEC serotypes have been described in the literature and a number are commercially available. However, the absence of clear-cut metabolic and antimicrobial resistance features amongst some of the non-O157 STEC strains makes the development of one single isolation medium for all STEC very challenging.

Profound studies on the broad variety of isolation media for STEC are still useful. Therefore in *chapter 3*, six selective isolation media have been evaluated. To ensure a rapid and reliable isolation method for routine laboratories the **colony morphology** should be **unambiguous** for all STEC. Both the USDA recommended Rainbow® agar O157 (RB) and the isolation medium developed by Possé *et al.* (2008) (mMac) displayed a variety of colors for the different STEC strains. While for both media the appearance of the different STEC serotypes has been described, the colony color is often ambiguous. Other studies also observed the lack of distinct colors

associated with certain STEC serotypes on RB, except for *E. coli* O157, which were blue-grey (Gill *et al.*, 2014; Windham *et al.*, 2013). Mathusa *et al.* (2010) noted that the color and entire aspect of the colonies on mMac were greatly dependent upon the incubation time, how crowded or isolated the colonies are, or the food matrix from which they were isolated.

The selective isolation agars, CHROMagar™ STEC, mMac and RAPID *E. coli* O157:H7, were unable to support the growth of a significant proportion of the **stx-positive *E. coli* strains**. Moreover, USDA recommends a supplementation of RB with novobiocin, cefixime and tellurite to exclude many non-STEC strains, but this leads to the inhibition of some susceptible STEC strains (Gill *et al.*, 2014; Jinneman *et al.*, 2012; Kase *et al.*, 2015). As shown in our and other studies, the inability to support certain STEC strains appeared to be linked to the absence of tellurite resistance genes, especially for CHROMagar™ STEC and mMac (Kase *et al.*, 2015; Tzschoppe *et al.*, 2012). On the other hand, all *stx*-positive *E. coli* strains were able to growth on the low selective or inclusive agars TBX, RB and ChromID™ EHEC (except one). However, these isolation media also supported the growth of **all stx-negative *E. coli* strains**, often with colony colors indistinguishable from STEC colonies. Whereas the selective media were able to inhibit the non-target organisms, except for the EPEC.

Neither our results nor the literature provide a single isolation medium suitable for all STEC. Since the selective isolation media are unsuitable for the isolation of certain STEC strains, its use should be paired with an inclusive medium capable of supporting a broader range of STEC strains.

### 1.3. Isolation procedure

In chapter 4 we aimed for a faster and more reliable detection and isolation method for STEC from food. After enrichment in either BPW or Brila the samples were inoculated in parallel on an inclusive and selective isolation medium and maximum five presumptive STEC colonies of each plate were isolated and confirmed. Cooley *et al.* (2013) reported a background microbiota as high as  $10^9$  CFU ml<sup>-1</sup> after enrichment in a non-selective enrichment medium. We observed after enrichment in either BPW or Brila, a total bacterial count as high as  $7.5 \times 10^8$  CFU ml<sup>-1</sup>. The performance of both isolation media was dependent on the **type of food matrix**. While this might be explained by the particular composition of the interfering background microbiota accompanying the different food matrices, the exact explanation of this finding remains unclear. The presumptive STEC colonies on the selective agar (CHROMagar™ STEC) resulted in fewer false positives after PCR confirmation compared the inclusive agar (ChromID™ EHEC). However, the recovery of the **tellurite sensitive** STEC strain could only be achieved using the latter isolation medium. Therefore, the **parallel use of both chromogenic media** resulted in an overall increased

recovery compared to the use of one medium. Kase *et al.* (2015) also recommended a isolation strategy based on the paired use of both inclusive and selective agars. While Brusa *et al.* (2016) investigated the use of three inclusive agars in parallel, which significantly enhanced the recovery rate, but was still incapable of identifying all STEC strains.

To enhance the sensitivity of the cultural isolation, several methods have been described, such as IMS. While **IMS** proved effective for the isolation of *E. coli* O157, multiple studies have shown that the same efficiency cannot be achieved for non-O157 STEC strains. The lower success rate was due to the lack of antibody specificity and the carry-over effect of non-target organisms (Conrad *et al.*, 2014; Cooley *et al.*, 2013; Verstraete *et al.*, 2010). The **USDA guidelines** for the detection and isolation of non-O157 STEC from meat products and carcass and environmental sponges (MLG 5B.05) recommends an **acid treatment procedure** after the IMS step prior to inoculation onto an isolation medium. This approach is based on the acid tolerance of *E. coli* in order to reduce the carry-over microbiota from the IMS beads and to ensure a more effective isolation step for all STEC serotypes (Fedio *et al.*, 2012; Tillman *et al.*, 2012; Yoshitomi *et al.*, 2012). Furthermore, Grant *et al.* (2004) showed the benefit of using an acid treatment procedure independent of IMS for the isolation of STEC. Similarly in our study, the recovery of STEC from the artificially contaminated samples was significantly enhanced by the implementation of an acid treatment procedure. Surprisingly for the isolation of STEC from **naturally contaminated stx-positive food samples** using only the acid treatment could not improve recovery. A combination of both acid treatment and direct plating was required. However, in this experiment only a selective isolation medium (CHROMagar™ STEC) was used. Moreover, the enriched samples were often cold stored for a period of time, due to normal analytical circumstances. These cold stressed STEC strains may become more susceptible to the acid treatment. Furthermore, a broader variety of STEC strains can be found in naturally contaminated samples with different biochemical characteristics (i.e., fermentative profile) and antibiotic sensitivities, compared with the four STEC strains used for the artificially contaminated samples.

Despite the results for the natural contaminated samples, we still propose the acid treatment procedure as a suitable alternative for IMS, especially for the detection and isolation of all STEC serogroups in food types with high levels of competing microbiota, such as **sprouted seeds**. Both Verstraete *et al.* (2010) and Margot *et al.* (2015) reported the inability to recovery STEC from sprouted seeds with an inoculation level below  $10^3$  CFU ml<sup>-1</sup>. Similarly, our results showed a very poor recovery (3%) from the sprouted soy samples. However, the implementation of an acid treatment procedure has remarkably improved the recovery (68%). Moreover, the effectiveness of the acid treatment procedure seemed to be linked to the efficiency of the enrichment phase.

After an efficient enrichment phase, the recovery was not improved with an additional acid treatment step. However, after a less efficient enrichment phase, due to interfering factors such as the presence of high levels of competing microbiota, the merit of an additional acid treatment procedure was made clear.

In an ongoing study, not included in this thesis, we attempted to further optimize the isolation of STEC from sprouted seeds. These were inoculated with low numbers of tellurite sensitive STEC cells. A selective enrichment method in BPW was investigated, including a pre-enrichment or resuscitation phase for 7 hours at 37°C and enrichment for 17 hours at 44°C, before inoculating the inclusive isolation medium, TBX. As noted in *chapter 2* the enrichment in BPW should guaranteed the best resuscitation and growth after both seven and 24 hours of enrichment, regardless of STEC strain or type of stress. Preliminary results showed an improved recovery compared to the ISO/TS 13136:2012, especially with the addition of the acid treatment procure. However, in general the recovery from sprouted seeds is still relatively low compared to other food matrices. Moreover, since only an inclusive isolation media was used, a higher number of presumptive positive colonies was again needed for confirmation testing to ensure a successful recovery. Still further research is required.

After the outbreak in Germany in 2011, not only the CEN ISO/TS 13136 was amended, but the European Commission also amended the Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs as regard to the **microbiological criteria for sprouted seeds**. It stipulates the absence of STEC serotypes O157, O26, O111, O103, O145 and O104 in 25 gram of sprouted seeds. However, the isolation from this type of food was often unsuccessful (European Commission, 2013). The limited number of serogroups included in this regulation may allow a repeat of a new emerging highly virulent STEC strain belonging to another serogroup. Moreover, routine laboratories will only be required to isolate STEC strains from samples positive for these serogroups. This may lead to a significant information reduction about other STEC serogroups and may hamper future outbreak investigations. Finally, the regulation only covers sprouted seeds, despite the presence of *E. coli* O104:H4 in sprouted fenugreek seeds, the next emerging highly virulent STEC strain may as easily be linked to another raw consumed fruit, vegetable or meat product.

In *chapters 2, 3 and 4* only non-O157 STEC strains were investigated, whereas the STEC serogroup O157 remains of great important. Eventhough the widely accepted ISO 16654:2001 is very effective for the detection of *E. coli* O157, the value of the ISO/TS 13136 for the detection of all STEC has been proven since its introduction in 2012. In the near future the latter norm will be revised, it may be advisable to optimize one methodology instead of two separate detection methods.

However, further research will be needed to investigate our proposed methodology for the detection of *E. coli* O157.

## **2. Public health surveillance**

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Article 14 of the regulation (EC) No 178/2002 states the food safety requirements and prohibits food being placed on the market if it is unsafe. Food is considered unsafe if it is injurious to health or unfit for human consumption (European Commission, 2002). However, as the detection and isolation methods for STEC improves a broader variety of STEC strains are being identified in food. While in the past all strains belonging to the same species or pathotype were considered equally virulent, we are now faced with the reality that many different genes and factors may define the virulence of each individual strain (Coombes *et al.*, 2011). The Stx production remains the primary virulence trait responsible for severe symptoms, many STEC strains do not cause any symptoms. Therefore, assessing the clinical and public health risks associated with the different STEC strains remain of utmost importance.

### **2.1. Not all STEC are created equal**

Since nothing in life is completely risk-free, all STEC strains possess some pathogenicity dependent on bacterial, host and environmental factors. Therefore, any STEC strain should be able to be categorized as either a low or a high health risk. Four decades ago pathogenic *E. coli*, such as EPEC, could only be distinguished from other *E. coli* by serotyping (Jafari *et al.*, 2012). This phenotypic characterization became a routine practice and proved a useful tool to classify other pathogenic *E. coli*, such as *E. coli* O157. The **seropathotype classification system** proposed by Karmali (2003) was a valuable method which linked the known STEC serotypes, both *E. coli* O157:H7 and non-O157:H7, to outbreaks, the occurrence of HC and HUS, and their frequency. However, this system has several limitations. While the capacity to cause HC and HUS are the main features to classify STEC, its ability to cause solely diarrhea is also a public health concern. Moreover, this system did not recognize STEC serotype O104:H4 as a virulent strain before the outbreak. Furthermore, since the publication of the seropathotype classification many serotypes have been added after outbreaks and HUS cases. New STEC serotypes continue to be found associated with severe disease, such as O80:H2 STEC, O73:H18 STEC and O59:H19 STEC (Bielaszewska *et al.*, 2004; Mariani-Kurkdjian *et al.*, 2014; Prager *et al.*, 2014). Therefore, this approach remains retrospective and will always leave the opportunity for other emerging STEC serotypes to cause problems in the future.

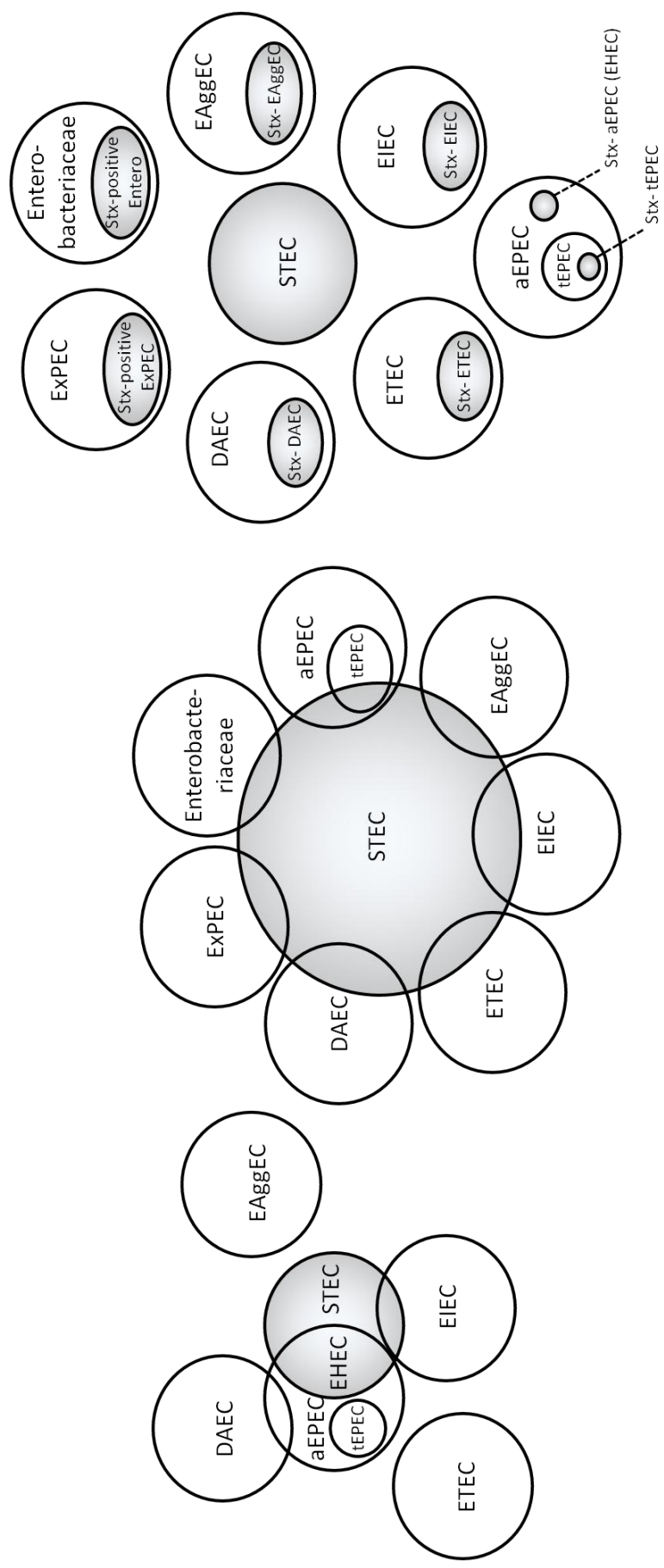
In the U.S. PFGE is routinely performed for enteric pathogens, including STEC. These PFGE patterns are uploaded to PulseNet and monitored by CDC for outbreak detection and

investigation (Caprioli *et al.*, 2014). While this has revolutionized outbreak detection, the resulting patterns provide limited information about the virulence of one particular strain. Therefore, the pathogenicity of STEC can only be determined by the **presence of *stx* and other virulence factors**.

Chapter 1 described the different *E. coli* pathotypes as separate entities. However, with the first STEC outbreak in 1982 this **dogma** was already challenged with the subgroup EHEC, which combined the virulence factors associated with STEC and EPEC (Figure VII-1: anno 2002). The more recent outbreak in Germany with the outbreak strain *E. coli* O104:H4, which combined the virulence factors associated with STEC and EAaggEC, challenged the original dogma even further. Moreover, recent research indicated that **all *E. coli* pathotypes are susceptible** for infection by Stx-phages (Schmidt *et al.*, 1999; Tozzoli *et al.*, 2014). However in contrast to STEC, these *E. coli* pathotypes have a human reservoir and are capable for inter-human transmission. This may have contributed to the severity of the *E. coli* O104:H4 outbreak and presents a considerable concern for future emerging hybrid STEC strains. Moreover, **extra-intestinal pathogenic *E. coli* and other *Enterobacteriaceae*** were also able to harbor a Stx-prophage (Paton and Paton, 1996; Wester *et al.*, 2013). Therefore, the original dogma concerning *E. coli* pathotypes was seriously revised (Figure VII-1: anno 2015).

Figure VII-1. The changing view regarding the relationships between *E. coli* pathotypes and other *Enterobacteriaceae* as described by Donnenberg (2002) and Morabito (2015) and a proposed alternative view on the latter. In this alternative view the different *E. coli* pathotypes and *Enterobacteriaceae* are again regarded as separate entities, each with their particular “virulence backbone”. Those organisms that also harbor *stx* genes are presented as a subset of each pathotype. DAEC: Diffuse-adhering *E. coli*, STEC: Shiga toxin-producing *E. coli*, EIEC: Enteroinvasive *E. coli*, tEPEC: typical Enteropathogenic *E. coli*, aEPEC: atypical Enteropathogenic *E. coli*, EHEC: Enterohaemorrhagic *E. coli*, ETEC: Enterotoxigenic *E. coli*, EAaggEC: Enteroaggregative *E. coli*, ExPEC: Extraintestinal pathogenic *E. coli*.





Alternative view

Anno 2015

Anno 2002

Still, this representation may be inaccurate. Therefore, a slight modification could be proposed that more or less restores the dogma of the separate entities (Figure VII-1: Alternative view). Piérard *et al.* (2012) proposed to simplify the many nomenclatures with regard to STEC by adding the virulence-associated properties as a suffix to the acronym STEC (e.g. AE-STEC and Agg-STEC). According to this nomenclature the hybrid *E. coli* O104:H4 strain is denoted as Agg-STEC. However, genetic characterization classified this hybrid strain as an EAggEC which acquired a Stx-phage. Therefore, the suffix of “Stx-” to the acronym EAggEC may be more accurate.

The resulting **alternative view** emphasizes the different “virulence backbones” derived from the different *E. coli* pathotypes that constitute the STEC population. In *chapter 5* we characterized a collection of STEC strains isolated from food and humans by determining the PFGE patterns and detecting the most common O- and H-type specific genes, pathotype specific genes, several virulence genes and an antimicrobial resistance gene. For these analyses a microbead-based suspension array (Luminex xMAP®) was applied after a limited number of multiplex PCR's. Using the combination of data from the PFGE patterns and virulence profile we were able to provide an indication regarding the public health risk of the different food isolates. While further research is certainly needed, techniques like the Luminex xMAP® (Lin *et al.*, 2011; Taniuchi *et al.*, 2012) or micro-array (Bugarel *et al.*, 2010; Lacher *et al.*, 2014), may ensure characterization of a STEC strain in a “single shot”, providing valuable information essential for the “**Molecular Risk Assessment**” (MRA). However, characterization is only initiated after detection and isolation of *stx*-positive *E. coli*. It is known that STEC strains may lose its Stx-phage in the course of an infection or during laboratory handling. These strains can potentially be re-infected with Stx-phages at a later moment. Still, these *stx*-positive and *stx*-negative *E. coli* are classified as different *E. coli* pathotypes. In *chapter 5*, 17 of the 35 aEPEC strains showed genetic relatedness to STEC strains with a higher virulence potential. Similarly, Trabulsi *et al.* (2002) noted that most of the aEPEC strains showed a closer genetic relationship with EHEC strains in comparison with tEPEC strains. While these aEPEC strains will not be detected by the ISO/TS 13136:2012, if re-infection with a Stx-phage should occur they may still become a public health risk. This clearly illustrates that the “**virulence backbone**” of any *E. coli* is of great importance besides the presence of the *stx* genes. Therefore we suggest a more extended characterization of a STEC isolate, not only the detection of the main virulence genes (e.g. *stx 1*, *stx 2*, *eae*) and serotyping, but a screening of the main virulence genes associated with all known diarrheagenic *E. coli* pathotypes (Figure VII-2). This approach should maximize the chance of detection future hybrid outbreak strains, such as *E. coli* O104:H4.

The STEC strains we characterized in *chapter 5* were isolated over a period from 2000 to 2014, using different detection and isolation methods. The chosen methods (e.g. the use of tellurite)

may have influenced the composition of the STEC strain collection and therefore our conclusion. The use of an acid treatment procedure or/and the use of a second isolation medium without tellurite may increase the number of successful isolations of a subset in the STEC population that may have been missed in the past. Whether the virulence profile of these strains will differ remains uncertain.

While the focus up to now was solely on the pathogen, other factors should be taken into account during the public health decision making. Article 14 of the regulation (EC) No 178/2002 is based on the assumption that food is always **consumed under normal conditions** (e.g. proper cooking). Moreover, different types of food are linked to different degrees of health risks. In accordance with the different STEC strains, the different types of food may be classified as low and **high health risk food**, such as certain ready-to-eat food. The European Commission published a guidance document for the application of article 14 in regard of STEC-positive food. This document proposes different corrective actions according to the level of risk associated with the contaminated food. For high health risk food the detection and isolation of a *stx*-positive *E. coli* may be sufficient to initiate a corrective action (Figure VII-2). On the other hand, for **low health risk food** the *stx*-positive *E. coli* should carry an additional colonizing factor, such as *eae* and *aa/C* (and its regulating gene *aggR*) and belong to the “big five” STEC serogroups before corrective actions are warranted (European Commission, 2014). While the economical impact of such an approach will have to be monitored, from the public health point of view it is a promising temperate approach until the right combination of markers to define pathogenic STEC are found and the detection techniques are further optimized.

Since recent techniques allow the detection of many different genes at once it may be advisable to include a routine **Stx subtyping** in the future. The routine characterization of Stx subtypes can be performed next to or instead of serotyping. The number of Stx subtypes is rather limited compared to the multitude of differed STEC serotypes and are strongly associated with the clinical importance (Scheutz *et al.*, 2012). This information in addition to the type of food and any other virulence factor may result in fast and well-informed public health decision (Figure VII-2, dotted line).

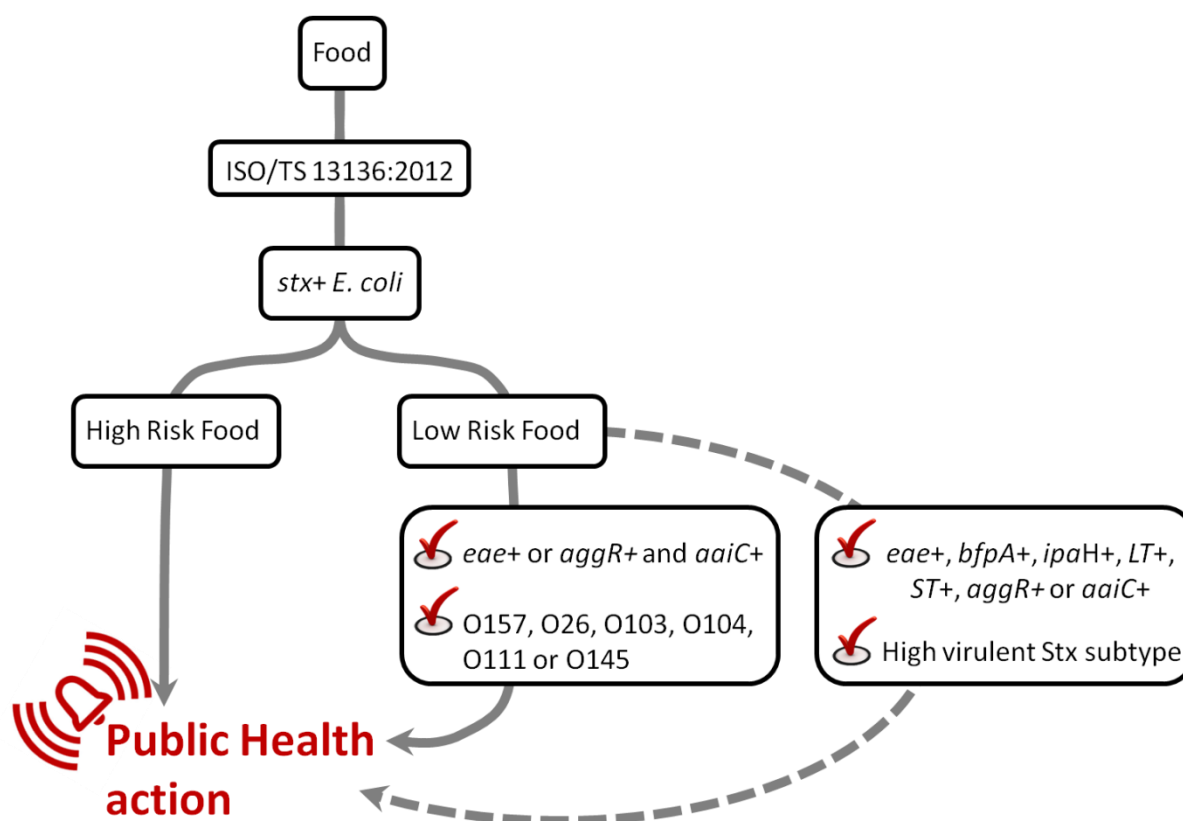


Figure VII-2 Flowchart with recommendations for public health actions in case STEC is detected in food, with regard to the foods risk profile and STEC characteristics as described in the guidance document for the application of article 14 of Regulation (EC) No 178/2002 (European Commission, 2014). The dotted line indicates a proposed alternative, replacing the serogroups with Stx subtyping and broadening the scope of virulence genes.

### 3. Quantification of STEC by “divide and conquer”

Ruminants, especially cattle, are regarded as the natural reservoir for STEC. In the last years qPCR is often applied to quantify STEC in cattle feces and provide answers to questions regarding STEC shedding, persistence and efficiency of treatments. However, this approach is based on relative quantification and totally dependent on the accuracy of the standard curve. Moreover there is a need for a DNA purification procedure to eliminate potential inhibitors able to interfere with the results. In order to overcome these shortcomings, a “third-generation PCR” or digital PCR (dPCR) has been developed.

In *chapter 6* the features of a qPCR, as described by Verstraete *et al.* (2014) were compared with the novel dPCR for the quantification of *stx1*, *stx2* and *eae* in cattle feces. The dPCR technique allows for absolute quantification of target DNA molecules without the requirement for a standard curve, based on the principle of “divide and conquer”. The sample is partitioned into hundreds or thousands of separate reaction chambers so that each contains either one or no copy of the target DNA. The absolute number of target DNA in the sample is calculated directly from the ratio of the positive to the total of partitions using binomial Poisson statistics. Two types of dPCR have been developed, the dPCR on chips and the droplet digital PCR (ddPCR). For

the **dPCR on chips** the sample will be partitioned into hundreds of chambers. In accordance with qPCR the fluorescence of each chamber will be measured after every successive cycle. In this regard the dPCR on chips is better equipped with amplification curves for every positive partition. Based on the target C<sub>q</sub> level, positive partitions can be distinguished from negative and even the false positive partitions by non-specific amplification (Dong *et al.*, 2015). On the other hand, the **ddPCR** will partition the sample in thousands of droplets and will only measure the fluorescence after completion of the amplification. This cheaper form of dPCR provides no information about the levels of target DNA over the course of amplification. For the comparison the already validated qPCR-based protocol as described by Verstraete *et al.* (2014) was used to optimize the ddPCR. **The optimization** is essential to prevent the appearance of a “smear” of droplets, in which positive droplets cannot be distinguished from negative droplets and no quantification can be performed.

The upper quantification limit for the ddPCR was notably lower in comparison to qPCR, due to saturation by positive droplets. The **limits of quantification (LOQ)** for both ddPCR and qPCR were similar. However, this LOQ is still higher (about 10<sup>3</sup> CFU g<sup>-1</sup>) compared to the culture-based approach (>10<sup>1</sup> CFU g<sup>-1</sup>). To increase the LOQ of STEC in cattle feces for qPCR an initial enrichment step of 5 hours was been suggested. With the use of a calibration curve the initial concentration can be calculated from the qPCR result (Lawal *et al.*, 2015; Shridhar *et al.*, 2016). The accuracy of the quantification is greatly dependent on calibration curve and the assumption that all STEC strains will show the same behavior during enrichment, which is certainly doubtful.

The dPCR is believed to be **less prone to PCR inhibition** compared to qPCR. During the latter assay, the inhibitors increase the number of amplification cycles required to reach a given threshold value. In contrast for ddPCR the number of cycles is unimportant, as long as the amplification is sufficient to separate the positive from the negative droplets. Therefore, inhibitors could still be a problem if they cause false negatives by preventing amplification from occurring at all (Baker, 2012). In *Chapter 6* both PCR techniques seemed capable of overcoming inhibition, although for qPCR a more **specialized mastermix** (EMM) appeared to be required. Besides the PCR technique and mastermix, we showed that the **sample type** is also of importance. A classical fecal sample has a heterogeneous composition and because only a very small volume will be subjected to DNA purification procedures a perfect homogenization is required. However, perfect homogenization is difficult to achieve, therefore low numbers of STEC can be missed. In addition, these samples may contain several components able to interfere with the amplification. On the other hand, the use of recto-anal-mucosal swab (RAMS) provides a sampling at one of the main colonization sites of STEC, resulting in much less material which is easier to homogenize. A higher positive rate was noted from RAMS in comparison to fecal

samples from the same animal. While some of the fecal samples contained PCR inhibitors, these seemed to be absent in the RAMS. Furthermore, the practice of taking RAMS is easier and faster compared to a fecal sample taken directly from the rectum. However, other studies have indicated that RAMS was less efficient compared to fecal samples for culture-based detection of *E. coli* O157 (Niu *et al.*, 2008; Williams *et al.*, 2014).

While the ddPCR assay showed very similar characteristics to the qPCR when using an appropriate specialized mastermix (e.g. EMM), the independence from any standardized reference material is still a major improvement. Any future studies on shedding and persistence of STEC in the bovine herds should consider the applications of ddPCR and the merits of RAMS.

#### **4. Conclusions and future prospects**

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The development of NGS is already in the progress of revolutionizing the classical outbreak investigations. However, these methods still require individual isolates and as long as cultural isolation remains the only tool that yields an isolate, the entire public health surveillance will continue to hinge on a successful isolation. In this doctoral thesis a detection and isolation method of human pathogenic STEC in food was developed and evaluated. The **cultural enrichment** and PCR-based detection of STEC in food was found effective, but with still some room for improvement regarding difficult food matrices, such as sprouted seeds. The lack of common biochemical features for all STEC hinders the development of an adequate **isolation medium**. Therefore, we proposed the paired use of a selective and inclusive isolation medium. To further improve the recovery rate we introduced an **acid treatment** prior to plating. While this treatment will not exclude other *E. coli*, the high background microbiota in food, such as sprouted soy beans, will be significantly reduced. These findings may play a role in the future revision of the ISO/TS 13136:2012. If this methodology is found to be effective for the detection of all STEC the discussion should be raised whether there is a need to maintain a separate detection method for *E. coli* O157. While this method has proven its effectiveness in the past, it is limited to the detection of non-sorbitol fermenting *E. coli* O157 and may be too selective for some stressed *E. coli* O157 (Jasson *et al.*, 2009; Vimont *et al.*, 2007). The isolated STEC strains can be **further characterized** based on PFGE patterns, serotype, diarrheagenic *E. coli* pathotype and virulence profile. However, these expensive and time-consuming conventional methods can only provide rather limited phylogenetic data. Therefore, work has started to replace these methods with those using **NGS** data, such as Whole Genome Sequencing (WGS) and single nucleotide polymorphisms (SNP). This technology will provide high resolution insights in the evolution of the STEC population and its divergence. It will further improve the understanding of transmission of STEC and its sources (Whittam and Bumbaugh, 2002). Serotyping, virulence and

antimicrobial resistance profiling can be performed in one single workflow within days. A clear example of its effectiveness was already shown during the German outbreak in 2011. Because of the increasing interest in NGS the cost has become less prohibitive and more accessible for many laboratories. However, the massive amount of generated sequences require appropriate bio-informatic processing, which may form a major bottleneck for non-specialists (Franz *et al.*, 2014; Vincent *et al.*, 2016). Moreover, close-related serogroups (e.g. O153 and O178) appear indistinguishable with WGS, while no cross-reactivity could be observed using the conventional phenotypic tests (DebRoy *et al.*, 2016). Furthermore, this raw genome sequence data needs to be translated to the **biology of the pathogen** that is useful for public health professionals. Therefore, suitable NGS analysis tools should be available to process high quality data and sufficient epidemiological context information (Lindsey *et al.*, 2016).

The ruminant population is still considered to be the main source of STEC. Therefore, we investigated the use of a third generation PCR, the droplet digital PCR, and RAMS as a novel method to quantify STEC in cattle. Whether for the PCR-based quantification of STEC in cattle feces or for the PCR-based detection of STEC in food, the DNA template consists of a mixture of DNA present in the original sample. Namely, the target genes may **originate from the same bacterium** or from different bacteria. In theory the ddPCR may solve this major bottleneck of the first and second generation PCR assays. Instead of partitioning the extracted DNA, **bacterial cells** should be partitioned into the separate reaction chambers so that each contains one or no cells. Since the Gram-negative bacteria can easily be lysed during the first step of any PCR protocol (at 94-95°C), the following amplification will actually provide information about the content of one cell. This application for the ddPCR may revolutionize STEC detection and any public health interventions.

The cattle reservoir remain the focus of most studies, as a result the **other ruminants and animals** may be overlooked. Animals, such as pigeons, have been reported to harbor *stx2f* STEC strains, but are no reservoir for human infection. However, they may act as a reservoir for the Stx2f-phage, that can infect *E. coli* and at their turn cause severe disease. This also highlights the importance of the prevalence and role of the Stx-phage, for which information and research is still somewhat lacking.





## References

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- Ackermann, H., 2001. Frequency of morphological phage descriptions in the year 2000. Brief review. *Arch. Virol.* 146, 843–857.
- Ahmed, N., Dobrindt, U., Hacker, J., Hasnain, S.E., 2008. Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. *Nat. Rev. Microbiol.* 6, 387–394.
- Ahmed, W., Gyawali, P., Toze, S., 2015. Quantitative PCR measurements of *Escherichia coli* including shiga toxin-producing *E. coli* (STEC) in animal feces and environmental waters. *Environ. Sci. Technol.* 49, 3084–3090.
- Aldick, T., Bielaszewska, M., Zhang, W., Brockmeyer, J., Schmidt, H., Friedrich, A.W., Kim, K.S., Schmidt, M.A., Karch, H., 2007. Hemolysin from Shiga toxin-negative *Escherichia coli* O26 strains injures microvascular endothelium. *Microbes Infect.* 9, 282–290.
- Aldus, C.F., Van Amerongen, A., Ariëns, R.M.C., Peck, M.W., Wichers, J.H., Wyatt, G.M., 2003. Principles of some novel rapid dipstick methods for detection and characterization of verotoxigenic *Escherichia coli*. *J. Appl. Microbiol.* 95, 380–389.
- Anderson, K.M., Abbott, J., Zhao, S., Liu, E., Himathongkham, S., 2015. Molecular Subtyping of Shiga Toxin-Producing *Escherichia coli* Using a Commercial Repetitive Sequence-Based PCR Assay. *J. Food Prot.* 78, 902–11.
- Aradská, J., Šmidák, R., Turkovičová, L., Turňa, J., Lubec, G., 2013. Proteomic differences between tellurite-sensitive and tellurite-resistant *E.coli*. *PLoS One* 8, 1–9.
- Archer, D.L., 2004. Freezing: an underutilized food safety technology? *Int. J. Food Microbiol.* 90, 127–38.
- Avery, L.M., Williams, A.P., Killham, K., Jones, D.L., 2008. Survival of *Escherichia coli* O157:H7 in waters from lakes, rivers, puddles and animal-drinking troughs. *Sci. Total Environ.* 389, 378–385.
- Bai, X., Wang, H., Xin, Y., Wei, R., Tang, X., Zhao, A., Sun, H., Zhang, W., Wang, Y., Xu, Y., Zhang, Z., Li, Q., Xu, J., Xiong, Y., 2015. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* isolated from retail raw meats in China. *Int. J. Food Microbiol.* 200, 31–38.
- Baker, M., 2012. Digital PCR hits its stride. *Nat. Methods* 9, 541–544.
- Balière, C., Rincé, A., Blanco, J., Dahbi, G., Harel, J., Vogeleer, P., Giard, J., Mariani-Kurkdjian, P., Gourmelon, M., 2015. Prevalence and Characterization of Shiga Toxin-Producing and Enteropathogenic *Escherichia coli* in Shellfish-Harvesting Areas and Their Watersheds. *Front. Microbiol.* 6, 1356.
- Barco, L., Belluco, S., Roccato, A., Ricci, A., 2015. A systematic review of studies on *Escherichia coli* and *Enterobacteriaceae* on beef carcasses at the slaughterhouse. *Int. J. Food Microbiol.* 207, 30–39.
- Baylis, C.L., 2008. Growth of pure cultures of Verocytotoxin-producing *Escherichia coli* in a range of enrichment media. *J. Appl. Microbiol.* 105, 1259–65.
- Beer, N.R., Hindson, B.J., Wheeler, E.K., Hall, S.B., Rose, K.A., Kennedy, I.M., Colston, B.W., 2007. Reaction in Picoliter Droplets. *Anal. Chem.* 79, 8471–8475.
- Benjamin, M.M., Datta, A.R., 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl. Environmental Microbiol.* 61, 1669–72.
- Berenger, B.M., Berry, C., Peterson, T., Fach, P., Delannoy, S., Li, V., Tschetter, L., Nadon, C., Honish, L., Louie, M., Chui, L., 2015. The utility of multiple molecular methods including whole genome sequencing as tools to differentiate *Escherichia coli* O157:H7 outbreaks. *Eurosurveillance* 20, 1–11.
- Bergholz, T.M., Whittam, T.S., 2007. Variation in acid resistance among enterohaemorrhagic *Escherichia coli* in a simulated gastric environment. *J. Appl. Microbiol.* 102, 352–62.
- Besse, G.N., Brissonnet, D.F., Lafarge, V., Leclerc, V., 2000. Effect of various environmental parameters on the recovery of sublethally salt-damaged and acid-damaged *Listeria monocytogenes*. *J. Appl. Microbiology* 89, 944–50.
- Bettelheim, K. a, Beutin, L., 2003. Rapid laboratory identification and characterization of verocytotoxigenic (Shiga toxin producing) *Escherichia coli* (VTEC/STEC). *J. Appl. Microbiol.* 95, 205–217.
- Bettelheim, K.A., 2005. Reliability of O157:H7 ID agar (O157 H7 ID-F) for the detection and isolation of verocytotoxigenic strains of *Escherichia coli* belonging to serogroup O157. *J. Appl. Microbiol.* 99, 408–410.
- Beutin, L., Fach, P., 2014. Detection of Shiga Toxin-Producing *Escherichia coli* from Nonhuman Sources and Strain Typing. *Microbiol. Spectr.* 2, 1–23.
- Beutin, L., Steinrück, H., Krause, G., Steege, K., Haby, S., Hultsch, G., Appel, B., 2007. Comparative evaluation of the Ridascreen® Verotoxin enzyme immunoassay for detection of Shiga-toxin producing strains of *Escherichia coli* (STEC) from food and other sources. *J. Appl. Microbiol.* 102, 630–639.
- Bhagwat, A.A., Chan, L., Han, R., Tan, J., Kothary, M., Jean-gilles, J., Tall, B.D., 2005. Characterization of Enterohemorrhagic *Escherichia coli* Strains Based on Acid Resistance Phenotypes. *Infect. Immun.* 73, 4993–5003.
- Bielaszewska, M., Fell, M., Greune, L., Prager, R., Fruth, A., Tschape, H., Schmidt, M.A., Karch, H., Tscha, H., 2004. Characterization of cytolethal distending toxin genes and expression in shiga toxin-producing *Escherichia coli* strains of non-O157

- serogroups. *Infect Immun* 72, 1812–1816.
- Bolton, D.J., 2011. Verocytotoxigenic (Shiga toxin-producing) *Escherichia coli* virulence factors and pathogenicity in the farm to fork paradigm. *Foodborne Pathog. Dis.* 8, 357–65.
- Bosilevac, J.M., Koohmaraie, M., 2011. Prevalence and characterization of non-O157 shiga toxin-producing *Escherichia coli* isolates from commercial ground beef in the United States. *Appl. Environ. Microbiol.* 77, 2103–2112.
- Botteldoorn, N., Heyndrickx, M., Rijpens, N., Herman, L., 2003. Detection and characterization of verotoxigenic *Escherichia coli* by a VTEC/EHEC multiplex PCR in porcine faeces and pig carcass swabs. *Res. Microbiol.* 154, 97–104.
- Brett, K.N., Ramachandran, V., Hornitzky, M. a, Bettelheim, K. a, Walker, M.J., Djordjevic, S.P., 2003. stx 1c is the Most Common Shiga Toxin 1 Subtype among Shiga Toxin-Producing *Escherichia coli* Isolates from Sheep but Not among Isolates from Cattle. *J. Clin. Microbiol.* 41, 926–936.
- Bridgwater, F.A., Morgan, R.S., Rowson, K.E., Wright, G.P., 1955. The neurotoxin of *Shigella shigae*: morphological and functional lesions produced in the central nervous system of rabbits. *Br. J. Exp. Pathol.* 36, 447–453.
- Brooks, J.T., Sowers, E.G., Wells, J.G., Greene, K.D., Griffin, P.M., Hoekstra, R.M., Strockbine, N. a, 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J. Infect. Dis.* 192, 1422–9.
- Brunner, W., Schmidt, H., Frosch, M., Karch, H., 1999. The large plasmids of Shiga-toxin-producing *Escherichia coli* (STEC) are highly variable genetic elements. *Microbiology* 145, 1005–1014.
- Brusa, V., Piñeyro, P.E., Galli, L., Linares, L.H., Ortega, E.E., Padola, N.L., Leotta, G.A., 2016. Isolation of Shiga Toxin-Producing *Escherichia coli* from Ground Beef Using Multiple Combinations of Enrichment Broths and Selective Agars. *Foodborne Pathog. Dis.* 13, 163–170.
- Bugarel, M., Beutin, L., Martin, A., Gill, A., Fach, P., 2010. Micro-array for the identification of Shiga toxin-producing *Escherichia coli* (STEC) seropathotypes associated with Hemorrhagic Colitis and Hemolytic Uremic Syndrome in humans. *Int. J. Food Microbiol.* 142, 318–29.
- Bugarel, M., Martin, A., Fach, P., Beutin, L., 2011. Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* strains: a basis for molecular risk assessment of typical and atypical EPEC strains. *BMC Microbiol.* 11, 1–10.
- Burgos, Y., Beutin, L., 2012. Evaluation of an Immuno-Chromatographic Detection System for Shiga Toxins and the *E. coli* O157 Antigen, in: Albuelzein, E. (Ed.), *Trends in Immunolabelled and Related Techniques*. pp. 29–40.
- Bustin, S. a, Nolan, T., 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.* 15, 155–66.
- Buven, G., Piérard, D., 2012. Virulence profiling and disease association of verocytotoxin-producing *Escherichia coli* O157 and non-O157 isolates in Belgium. *Foodborne Pathog. Dis.* 9, 530–535.
- Caprioli, A., Morabito, S., Brugère, H., Oswald, E., 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Vet. Res.* 36, 289–311.
- Caprioli, A., Scavia, G., Morabito, S., 2014. Public Health Microbiology of Shiga Toxin-Producing *Escherichia coli*. *Microbiol. Spectr.* 2, 1–12.
- Castro-Rosas, J., Cerna-Cortés, J.F., Méndez-Reyes, E., Lopez-Hernandez, D., Gómez-Aldapa, C. a, Estrada-Garcia, T., 2012. Presence of faecal coliforms, *Escherichia coli* and diarrheagenic *E. coli* pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. *Int. J. Food Microbiol.* 156, 176–80.
- Catarama, T.M.G., O'Hanlon, K. a, Duffy, G., Sheridan, J.J., Blair, I., McDowell, D. a, 2003. Optimization of enrichment and plating procedures for the recovery of *Escherichia coli* O111 and O26 from minced beef. *J. Appl. Microbiol.* 95, 949–57.
- CDC, 2016. Reports of *E. coli* Outbreak Investigations from 2016. URL <http://www.cdc.gov/ecoli/2016-outbreaks.html> (accessed 3.23.16).
- CDC, 2015. Reports of *E. coli* Outbreak Investigations from 2015. URL <http://www.cdc.gov/ecoli/2015-outbreaks.html> (accessed 3.23.16).
- CDC, 2014. Reports of *E. coli* Outbreak Investigations from 2014. URL <http://www.cdc.gov/ecoli/2014-outbreaks.html> (accessed 3.23.16).
- CDC, 2013. Reports of *E. coli* Outbreak Investigations from 2013. URL <http://www.cdc.gov/ecoli/2013-outbreaks.html> (accessed 3.23.16).
- CDC, 2012. Reports of *E. coli* Outbreak Investigations from 2012. URL <http://www.cdc.gov/ecoli/2012-outbreaks.html> (accessed 3.23.16).
- CDC, 2011. Reports of *E. coli* Outbreak Investigations from 2011. URL <http://www.cdc.gov/ecoli/2011-outbreaks.html> (accessed 3.23.16).
- Chapman, P., Siddons, C., Zadik, P.M., Jewes, L., 1991. An improved selective medium for the isolation of *Escherichia coli* O157. *J. Med. Microbiol.* 35, 107–10.
- Chapman, P.A., Ellin, M., Ashton, R., Shafique, W., 2001. Comparison of culture, PCR and immunoassays for detecting *Escherichia coli* O157 following enrichment culture and immunomagnetic separation performed on naturally contaminated raw meat products. *Int. J. Food Microbiol.* 68, 11–20.

- Charsteen, T.G., Fuentes, D.E., Tantalean, J.C., Vasquez, C.C., 2009. Tellurite: history, oxidative stress, and molecular mechanisms of resistance. *FEMS Microbiol. Rev.* 33, 820–832.
- Chase-Topping, M., Gally, D., Low, C., Matthews, L., Woolhouse, M., 2008. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat. Rev. Microbiol.* 6, 904–12.
- Chaudhuri, R.R., Henderson, I.R., 2012. The evolution of the *Escherichia coli* phylogeny. *Infect. Genet. Evol.* 12, 214–226.
- Chiang, S.-K., Lou, Y.-C., Chen, C., 2008. NMR solution structure of KP-TerB, a tellurite-resistance protein from *Klebsiella pneumoniae*. *Protein Sci.* 17, 785–9.
- Chui, L., Couturier, M.R., Chiu, T., Wang, G., Olson, A.B., McDonald, R.R., Antonishyn, N. a, Horsman, G., Gilmour, M.W., 2010. Comparison of Shiga toxin-producing *Escherichia coli* detection methods using clinical stool samples. *J. Mol. Diagnostics* 12, 469–475.
- Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* 66, 4555–4558.
- Conrad, C.C., Stanford, K., McAllister, T.A., Thomas, J., Reuter, T., 2014. Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces. *J. Microbiol. Methods* 105, 22–30.
- Cooley, M.B., Jay-Russell, M., Atwill, E.R., Carychao, D., Nguyen, K., Quiñones, B., Patel, R., Walker, S., Swimley, M., Pierre-Jerome, E., Gordus, A.G., Mandrell, R.E., 2013. Development of a robust method for isolation of shiga toxin-positive *Escherichia coli* (STEC) from fecal, plant, soil and water samples from a leafy greens production region in California. *PLoS One* 8, e65716.
- Coombes, B.K., Gilmour, M.W., Goodman, C.D., 2011. The evolution of virulence in non-O157 shiga toxin-producing *Escherichia coli*. *Front. Microbiol.* 2, 1–3.
- Coombes, B.K., Wickham, M.E., Mascarenhas, M., Gruenheid, S., Finlay, B.B., Karmali, M. a, 2008. Molecular analysis as an aid to assess the public health risk of non-O157 Shiga toxin-producing *Escherichia coli* strains. *Appl. Environ. Microbiol.* 74, 2153–60.
- Cowley, L.A., Beckett, S.J., Chase-Topping, M., Perry, N., Dallman, T.J., Gally, D.L., Jenkins, C., 2015. Analysis of whole genome sequencing for the *Escherichia coli* O157:H7 typing phages. *BMC Genomics* 16, 271.
- Cronquist, A.B., Mody, R.K., Atkinson, R., Besser, J., D'Angelo, M.T., Hurd, S., Robinson, T., Nicholson, C., Mahon, B.E., 2012. Impacts of culture-independent diagnostic practices on public health surveillance for bacterial enteric pathogens. *Clin. Infect. Dis.* 54, 432–439.
- Davis, M.A., Rice, D.H., Sheng, H., Hancock, D.D., Thomas, E.B., Cobbold, R., Hovde, C.J., Besser, T.E., 2006. Comparison of cultures from rectoanal-junction mucosal swabs and feces for detection of *Escherichia coli* O157 in dairy heifers. *Appl. Environ. Microbiol.* 72, 3766–3770.
- De Boer, E., Heuvelink, A.E., 2000. Methods for the detection and isolation of Shiga toxin-producing *Escherichia coli*. *J. Appl. Microbiol.* 88, 1335–1435.
- De Rauw, K., Breynaert, J., Piérard, D., 2016. Evaluation of the Alere SHIGA TOXIN QUIK CHEK™ in comparison to multiplex Shiga toxin PCR. *Diagn. Microbiol. Infect. Dis.* 1–5.
- DebRoy, C., Fratamico, P.M., Yan, X., Baranzoni, G.M., Liu, Y., Needleman, D.S., Tebbs, R., O'Connell, C.D., Allred, A., Swimley, M., Mwangi, M., Kapur, V., Raygoza Garay, J.A., Roberts, E.L., Katani, R., 2016. Comparison of O-antigen gene clusters of all O-serogroups of *Escherichia coli* and proposal for adopting a new nomenclature for O-typing. *PLoS One* 11, 1–13.
- Debroy, C., Roberts, E., Kundrat, J., Davis, M.A., Briggs, C.E., Fratamico, P.M., 2004. Detection of *Escherichia coli* Serogroups O26 and O113 by PCR Amplification of the *wzx* and *wzy* Genes. *Appl. Environ. Microbiol.* 70, 1830–1832.
- Deisingh, a. K., Thompson, M., 2004. Strategies for the detection of *Escherichia coli* O157:H7 in foods. *J. Appl. Microbiol.* 96, 419–29.
- Delannoy, S., Beutin, L., Fach, P., 2012. Use of clustered regularly interspaced short palindromic repeat sequence polymorphisms for specific detection of enterohemorrhagic *Escherichia coli* strains of serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28, and O157:H7 by real-time. *J. Clin. Microbiol.* 50, 4035–4040.
- Doi, H., Takahara, T., Minamoto, T., Matsushashi, S., Uchii, K., Yamanaka, H., 2015. Droplet digital polymerase chain reaction (PCR) outperforms real-time PCR in the detection of environmental DNA from an invasive fish species. *Environ. Sci. Technol.* 49, 5601–8.
- Dong, Cho, H., 2014. Science Development of a multiplex loop-mediated isothermal amplification assay to detect shiga toxin-producing *Escherichia coli* in cattle. *J. Vet. Sci.* 15, 317–325.
- Dong, L., Meng, Y., Sui, Z., Wang, J., Wu, L., Fu, B., 2015. Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material. *Nat. Publ. Gr.* 2015.
- Donnenberg, M.S., 2002. Introduction, in: *Escherichia coli* Virulence Mechanism of a Versatile Pathogen. pp. XXI–XXV.

- Doyle, M.P., Schoeni, J.L., 1987. fresh meats and poultry . Isolation of *Escherichia coli* O157:H7 from Retail Fresh Meats and Poultry. Appl. Environ. Microbiol. 53, 2394–96.
- Druggan, P., Iversen, C., 2014. Chromogenic Agars, in: Batt, C.A., Tortorello, M.-L. (Eds.), Encyclopedia of Food Microbiology. pp. 248–258.
- Duffy, G., Burgess, C.M., Bolton, D.J., 2014. A review of factors that affect transmission and survival of verocytotoxigenic *Escherichia coli* in the European farm to fork beef chain. Meat Sci. 97, 375–83.
- Duffy, G., Walsh, C., Blair, I.S., McDowell, D. a, 2006. Survival of antibiotic resistant and antibiotic sensitive strains of *E. coli* O157 and *E. coli* O26 in food matrices. Int. J. Food Microbiol. 109, 179–86.
- Dumke, R., Schröter-Bobsin, U., Jacobs, E., Röske, I., 2006. Detection of phages carrying the Shiga toxin 1 and 2 genes in waste water and river water samples. Lett. Appl. Microbiol. 42, 48–53.
- Dykes, G., 2006. Laboratory-based simulation of freezing profiles of beef trim for *Escherichia coli* O157 survival determinations. J. Microbiol. Methods 64, 266–74.
- Eden, R., 2014. Classical and Modern Methods for Detection and Enumeration, in: Batt, C.A., Tortorello, M.-L. (Eds.), Encyclopedia of Food Microbiology. pp. 667–673.
- EFSA, 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA J. 13, 4329.
- EFSA, 2015. The EU Summary Report on Trends and Sources of Zoonoses , Zoonotic Agents and Food-borne Outbreaks in 2013.
- EFSA, 2014. The EU Summary Report on Trends and Sources of Zoonoses , Zoonotic Agents and Food-borne Outbreaks in 2012.
- EFSA, 2013a. The EU Summary Report on Trends and Sources of Zoonoses , Zoonotic Agents and Food-borne Outbreaks in 2011, EFSA Journal.
- EFSA, 2013b. Scientific Opinion on VTEC-seropathotype and scientific criteria regarding. EFSA J. 11.
- EFSA, 2013c. European Centre for Disease Prevention and Control; The European Union Summary Report on Trends and Sources of Zoonoses , Trends and Sources of Zoonoses , Zoonotic Agents and Food-borne Outbreaks in 2011, EFSA Journal 2013. d
- Elhanafi, D., Leenanon, B., Bang, W., Drake, M. a, 2004. Impact of cold and cold-acid stress on poststress tolerance and virulence factor expression of *Escherichia coli* O157:H7. J. Food Prot. 67, 19–26.
- Erickson, M.C., Doyle, M.P., 2007. Food as a vehicle for transmission of Shiga toxin-producing *Escherichia coli*. J. Food Prot. 70, 2426–49.
- Escherich, T., 1885. Die Darmbakterien des Neugeborenen und Säuglings. Fortschritte der Med. 3, 516–522.
- European Commission, 2014. Guidance document on the application of article 14 of Regulation (EC) n° 178/2002 as regards food where Shiga toxin-producing *Escherichia coli* (STEC) has been detected.
- European Commission, 2013. Commission Regulation (EU) No 209/2013 of 11 March 2013 amending Regulation (EC) No 2073/2005 as regards microbiological criteria for sprouts and the sampling rules for poultry carcasses and fresh poultry meat.
- European Commission, 2002. Regulation (EC) N° 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, Official Journal of the European Communities.
- Evans, J., Knight, H.I., Smith, a W., Pearce, M.C., Hall, M., Foster, G., Low, J.C., Gunn, G.J., 2008. Cefixime-tellurite rhamnose MacConkey agar for isolation of Vero cytotoxin-producing *Escherichia coli* serogroup O26 from Scottish cattle and sheep faeces. Lett. Appl. Microbiology 47, 148–52.
- Fach, P., Perelle, S., Dilasser, F., Grout, J., 2001. Comparison between a PCR-ELISA test and the vero cell assay for detecting Shiga toxin-producing *Escherichia coli* in dairy products and characterization of virulence traits of the isolated strains. J. Appl. Microbiol. 90, 809–818.
- Fagan, P.K., Hornitzky, M.A., Bettelheim, K.A., Djordjevic, S.P., 1999. Detection of Shiga-Like Toxin (*stx1* and *stx2*), Intimin (*eaeA*), and Enterohemorrhagic *Escherichia coli* (EHEC) Hemolysin (EHEC *hlyA*) Genes in Animal Feces by Multiplex PCR. Appl. Environ. Microbiol. 65, 868–72.
- Farfan, M.J., Torres, A.G., 2012. Molecular mechanisms that mediate colonization of shiga toxin-producing *Escherichia coli* strains. Infect. Immun. 80, 903–913.
- Fedio, W.M., Jinneman, K.C., Yoshitomi, K.J., Zapata, R., Weagant, S.D., 2012. Efficacy of a post enrichment acid treatment for isolation of *Escherichia coli* O157:H7 from alfalfa sprouts. Food Microbiol. 30, 83–90.
- Feng, L., Senchenkova, S.N., Tao, J., Shashkov, A.S., Liu, B., Shevelev, S.D., Reeves, P.R., Xu, J., Knirel, Y.A., Wang, L., 2005. Structural and Genetic Characterization of Enterohemorrhagic *Escherichia coli* O145 O Antigen and Development of an O145 Serogroup-Specific PCR Assay. J. Bacteriol. 187, 758–764.
- Feng, P., 2014. Shiga Toxin-Producing *Escherichia coli* (STEC) in Fresh Produce – A Food Safety Dilemma. Microbiol. Spectr. 2, 1–11.
- Ferdous, M., Zhou, K., Mellmann, A., Morabito, S.,

- Croughs, P.D., Boer, R.F. De, Kooistra-smid, A.M.D., Rossen, J.W.A., Friedrich, A.W., 2015. Is Shiga Toxin-Negative *Escherichia coli* O157:H7 Enteropathogenic or Enterohemorrhagic *Escherichia coli*? Comprehensive Molecular Analysis Using Whole-Genome Sequencing. *J. Clinical Microbiol.* 53, 3530–3538.
- Fogg, P.C.M., Saunders, J.R., McCarthy, A.J., Allison, H.E., 2012. Cumulative effect of prophage burden on Shiga toxin production in *Escherichia coli*. *Microbiology* 158, 488–497.
- Franz, E., Delaquis, P., Morabito, S., Beutin, L., Gobius, K., Rasko, D. a, Bono, J., French, N., Osek, J., Lindstedt, B.-A., Muniesa, M., Manning, S., LeJeune, J., Callaway, T., Beatson, S., Eppinger, M., Dallman, T., Forbes, K.J., Aarts, H., Pearl, D.L., Gannon, V.P.J., Laing, C.R., Strachan, N.J.C., 2014. Exploiting the explosion of information associated with whole genome sequencing to tackle Shiga toxin-producing *Escherichia coli* (STEC) in global food production systems. *Int. J. Food Microbiol.* 187, 57–72.
- Franz, E., Van Hoek, A.H.A.M., Wuite, M., Van Der Wal, F.J., De Boer, A.G., Bouw, E.I., Aarts, H.J.M., 2015. Molecular hazard identification of non-O157 Shiga toxin-producing *Escherichia coli* (STEC). *PLoS One* 10, 1–21.
- Fratamico, P.M., Debroy, C., Strobaugh, T.P., Chen, C.Y., 2005. DNA sequence of the *Escherichia coli* O103 O antigen gene cluster and detection of enterohemorrhagic *E. coli* O103 by PCR amplification of the *wzx* and *wzy* genes. *Can. J. Microbiol.* 51, 515–22.
- Friesema, I., van der Zwaluw, K., Schuurman, T., Kooistra-Smid, M., Franz, E., van Duynhoven, Y., van Pelt, W., 2014. Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011. *Euro Surveill.* 19, 26–32.
- Fukushima, H., Hoshina, K., Gomyoda, M., 2000. Selective isolation of *eae*-positive strains of shiga toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* 38, 1684–1687.
- Fuller, C.A., Pellino, C.A., Flagler, M.J., Strasser, J.E., Weiss, A.A., 2011. Shiga toxin subtypes display dramatic differences in potency. *Infect. Immun.* 79, 1329–1337.
- Garred, O., Van Deurs, B., Sandvig, K., 1995. Furin-induced cleavage and activation of shiga toxin. *J. Biol. Chem.* 270, 10817–10821.
- Gerner-Smidt, P., Hise, K., Kincaid, J., Hunter, S., Rolando, S., Hyytia-trees, E., Ribot, E.M., Swaminithan, B., 2006. PulseNet USA: A Five-Year Update. *Foodborne Pathog. Dis.* 3, 9–19.
- Gill, A., Huszczyński, G., Gauthier, M., Blais, B., 2014. Evaluation of eight agar media for the isolation of shiga toxin-producing *Escherichia coli*. *J. Microbiol. Methods* 96, 6–11.
- Gill, A., Martinez-Perez, A., McIlwham, S., Blais, B., 2012. Development of a method for the detection of verotoxin-producing *Escherichia coli* in food. *J. Food Prot.* 75, 827–37. doi:10.4315/0362-028X.JFP-11-395
- Gilmour, M.W., Olson, A.B., Andrysiak, A.K., Ng, L.K., Chui, L., 2007. Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing *Escherichia coli* serogroup lineages. *J. Med. Microbiol.* 56, 620–628.
- Girardeau, J.P., Dalmasso, A., Bertin, Y., Ducrot, C., Bord, S., Livrelli, V., Vernozy-Rozand, C., Martin, C., 2005. Association of virulence genotype with phylogenetic background in comparison to different serotypes of Shiga toxin-producing *Escherichia coli* isolates. *J. Clin. Microbiol.* 43, 6098–6107.
- Giron, J.A., Torres, A.G., Freer, E., Kaper, J.B., 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol. Microbiol.* 44, 361–379.
- Gonthier, a, Guérin-Fauble, V., Tilly, B., Delignette-Muller, M.L., 2001. Optimal growth temperature of O157 and non-O157 *Escherichia coli* strains. *Lett. Appl. Microbiol.* 33, 352–6.
- Grant, M. a., 2004. Improved Laboratory Enrichment for Enterohemorrhagic *Escherichia coli* by Exposure to Extremely Acidic Conditions. *Appl. Environ. Microbiol.* 70, 1226–30.
- Grant, M.A., Mogler, M.A., Harris, D.L., 2009. Comparison of Enrichment Procedures for Shiga Toxin – Producing *Escherichia coli* in Wastes from Commercial Swine Farms 3. *J. Food Prot.* 72, 1982–86.
- Griandomenico, A., Cerniglia, G., Biaglow, J., Stevens, C., Koch, C., 1997. The importance of sodium pyruvate in assessing damage produced by hydrogen peroxide. *Free Radic. Biol. Med.* 23, 426–34.
- Gyles, C.L., 2007. Shiga toxin-producing *Escherichia coli*: an overview. *J. Anim. Sci.* 85, E45–62.
- Hacker, J., Kaper, J.B., 2010. Pathogenicity Islands and the Evolution of Microbes. *Annu. Rev. Microbiol.* 54, 641–679.
- Harada, T., Iguchi, A., Iyoda, S., Seto, K., Taguchi, M., Kumeda, Y., 2015. Multiplex Real-Time PCR Assays for Screening of Shiga Toxin 1 and 2 Genes, Including All Known Subtypes, and *Escherichia coli* O26-, O111-, and O157-Specific Genes in Beef and Sprout Enrichment Cultures. *J. Food Prot.* 78, 1800–1811. d
- Hara-Kudo, Y., Ikeda, M., Kodaka, H., Nakagawa, K., Goto, K., Masuda, T., Konuma, H., Kojima, T., Kumagai, S., 2000a. Selective Enrichment with a Resuscitation Step for Isolation of Freeze-Injured *Escherichia coli* O157:H7 from Foods. *Appl. Environ. Microbiol.* 66, 2866–72.

- Hara-Kudo, Y., Ikeda, M., Komatsu, O., Yamamoto, S., Kumagai, S., 2002. Evaluation of a chromogenic agar medium for isolation of *Escherichia coli* O26. *Food Control* 13, 377–79.
- Hara-Kudo, Y., Konishi, N., Ohtsuka, K., Iwabuchi, K., Kikuchi, R., Isobe, J., Yamazaki, T., Suzuki, F., Nagai, Y., Yamada, H., Tanouchi, A., Mori, T., Nakagawa, H., Ueda, Y., Terajima, J., 2016. An interlaboratory study on efficient detection of Shiga toxin-producing *Escherichia coli* O26, O103, O111, O121, O145, and O157 in food using real-time PCR assay and chromogenic agar. *Int. J. Food Microbiol.* 230, 81–88.
- Hara-Kudo, Y., Konuma, H., Nakagawa, H., Kumagai, S., 2000b. *Escherichia coli* O26 detection from foods using an enrichment procedure and an immunomagnetic separation method. *Lett. Appl. Microbiol.* 30, 151–4.
- Haugum, K., Brandal, L.T., Lindstedt, B., Westera, L., Bergh, K., Afset, J.E., 2014. PCR based detection of Shiga toxin-producing *Escherichia coli* (STEC) in a routine microbiology laboratory over 16 years: molecular characterization of strains. *J. Clin. Microbiol.* 52, 3156–3163.
- He, X., Qi, W., Quiñones, B., McMahon, S., Cooley, M., Mandrell, R.E., 2011. Sensitive detection of Shiga toxin 2 and some of its variants in environmental samples by a novel immuno-PCR assay. *Appl. Environ. Microbiol.* 77, 3558–3564.
- Himathongkham, S., Dodd, M.L.E.E., Yee, J.K., Lau, D.K., Bryant, R.G., Badoiu, A.S., Lau, H.K., Guthertz, L.S., Crawford-miksza, L., Soliman, M.A., 2007. Recirculating Immunomagnetic Separation and Optimal Enrichment Conditions for Enhanced Detection and Recovery of Low Levels of *Escherichia coli* O157:H7 from Fresh Leafy Produce and Surface Water. *J. Food Prot.* 70, 2717–24.
- Hindson, C.M., Chevillet, J.R., Briggs, H.A., Gallichotte, E.N., Ruf, I.K., Hindson, B.J., Vessella, R.L., Tewari, M., 2013. Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat. Methods* 10, 1003–1005.
- Hiramatsu, R., Matsumoto, M., Miwa, Y., Suzuki, Y., 2002. Characterization of Shiga Toxin-Producing *Escherichia coli* O26 Strains and Establishment of Selective Isolation Media for These Strains. *J. Clin. Microbiol.* 40, 922–5.
- Hiramatsu, R., Matsumoto, M., Sakae, K., Miyazaki, Y., 2005. Ability of Shiga Toxin-Producing *Escherichia coli* and *Salmonella* spp. To Survive in a Desiccation Model System and in Dry Foods. *Appl. Environ. Microbiol.* 71, 6657 – 6663.
- Hirvonen, J.J., Siitonen, A., Kaukoranta, S.-S., 2012. Usability and performance of CHROMagar STEC medium in detection of Shiga toxin-producing *Escherichia coli* strains. *J. Clin. Microbiol.* 50, 3586–90.
- Hoey, D.E.E., Currie, C., Else, R.W., Nutikka, A., Lingwood, C.A., Gally, D.L., Smith, D.G.E., 2002. Expression of receptors for verotoxin 1 from *Escherichia coli* O157 on bovine intestinal epithelium. *J. Med. Microbiol.* 51, 143–149.
- Hornitzky, M.A., Bettelheim, K.A., Djordjevic, S.P., 2001. The detection of Shiga toxin-producing *Escherichia coli* in diagnostic bovine faecal samples using vancomycin-cefixime-cefsulodin blood agar and PCR. *FEMS Microbiol. Lett.* 198, 17–22.
- Hou, Z., Fink, R.C., Sugawara, M., Diez-Gonzalez, F., Sadowsky, M.J., 2013. Transcriptional and functional responses of *Escherichia coli* O157:H7 growing in the lettuce rhizoplane. *Food Microbiol.* 35, 136–42.
- Huggett, J.F., Novak, T., Garson, J.A., Green, C., Morris-jones, S.D., Miller, R.F., Zumla, A., 2008. Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. *BMC Res. Notes* 1, 1–9.
- Hull, A.E., Acheson, D.W., Echeverria, P., Donohue-Rolfe, A., Keusch, G.T., 1993. Mitomycin immunoblot colony assay for detection of Shiga-like toxin-producing *Escherichia coli* in fecal samples: comparison with DNA probes. *J. Clin. Microbiol.* 31, 1167–1172.
- Humphries, R.M., Griener, T.P., Vogt, S.L., Mulvey, G.L., Raivio, T., Donnenberg, M.S., Kitov, P.I., Surette, M., Armstrong, G.D., 2010. N-acetylglucosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic *Escherichia coli*. *Mol. Microbiol.* 76, 1111–1126.
- Hussein, H.S., Bollinger, L.M., 2008. Influence of selective media on successful detection of Shiga toxin-producing *Escherichia coli* in food, fecal, and environmental samples. *Foodborne Pathog. Dis.* 5, 227–244.
- Hussein, H.S., Bollinger, L.M., 2005. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. *J. Food Prot.* 68, 2224–41.
- Hussein, H.S., Bollinger, L.M., Hall, M.R., 2008. Growth and enrichment medium for detection and isolation of Shiga toxin-producing *Escherichia coli* in cattle feces. *J. Food Prot.* 71, 927–933.
- Hussein, H.S., Sakuma, T., 2005. Prevalence of shiga toxin-producing *Escherichia coli* in dairy cattle and their products. *J. Dairy Sci.* 88, 450–465.
- Imamovic, L., Muniesa, M., 2011. Quantification and evaluation of infectivity of shiga toxin-encoding bacteriophages in beef and salad. *Appl. Environ. Microbiol.* 77, 3536–3540.
- International Organization for Standardization (ISO), 2012. Microbiology of food and animal feed-Real-time PCR-based method for the detection of foodborne pathogen - Horizontal method for the detection of STEC and the determination of O157, O111, O26, O103 and O145 serogroups



- (ISO/TS 13136:2012). Geneva, Switzerland.
- International Organization for Standardization (ISO), 2001. ISO 16654:2001 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Escherichia coli* O157.
- Jafari, A., Aslani, M.M., Bouzari, S., 2012. *Escherichia coli*: A brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. Iran. J. Microbiol. 4, 102–117.
- Jasson, V., Rajkovic, A., Baert, L., Debevere, J., Uyttendaele, M., 2009. Comparison of enrichment conditions for rapid detection of low numbers of sublethally injured *Escherichia coli* O157 in food. J. Food Prot. 72, 1862–8.
- Jasson, V., Uyttendaele, M., Rajkovic, A., Debevere, J., 2007. Establishment of procedures provoking sub-lethal injury of *Listeria monocytogenes*, *Campylobacter jejuni* and *Escherichia coli* O157 to serve method performance testing. Int. J. Food Microbiol. 118, 241–9.
- Jerse, a E., Yu, J., Tall, B.D., Kaper, J.B., 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. U. S. A. 87, 7839–7843.
- Jinneman, K.C., Waite-Cusic, J.G., Yoshitomi, K.J., 2012. Evaluation of shiga toxin-producing *Escherichia coli* (STEC) method for the detection and identification of STEC O104 strains from sprouts. Food Microbiol. 30, 321–8.
- Johannes, L., Römer, W., 2010. Shiga toxins-from cell biology to biomedical applications. Nat. Rev. Microbiol. 8, 105–116.
- Johnson, R.P., Clarke, R.C., Wilson, J.B., Read, S.C., Rahn, K., Renwick, S.A., Sandhu, K.A., Alves, D., Karmali, M.A., Lior, H., McEwen, S.A., Spika, J.S., Gyles, C.L., 1996. Growing Concerns and Recent Outbreaks Involving Non-O157:H7 Serotypes of Verotoxigenic *Escherichia coli*. J. Food Prot. 59, 1112–1122.
- Joris, M.A., Verstraete, K., de Reu, K., de Zutter, L., 2011. Loss of vtx genes after the first subcultivation step of verocytotoxigenic *Escherichia coli* O157 and non-O157 during isolation from naturally contaminated fecal samples. Toxins (Basel). 3, 672–677.
- Ju, W., Rump, L., Toro, M., Shen, J., Cao, G., Zhao, S., Meng, J., 2014. Pathogenicity Islands in Shiga Toxin-Producing *Escherichia coli* O26, O103, and O111 Isolates from Humans and Animals. Foodborne Pathog. Dis. 11, 342–345.
- Juillot, S., Römer, W., 2014. Shiga Toxins, in: Morabito, S. (Ed.), Pathogenic *Escherichia Coli* Molecular and Cellular Microbiology. pp. 79–102.
- Kalchayanand, N., Arthur, T.M., Bosilevac, J.M., Wells, J.E., Wheeler, T.L., 2013. Chromogenic agar medium for detection and isolation of *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from fresh beef and cattle feces. J. Food Prot. 76, 192–9.
- Kanki, M., Seto, K., Harada, T., Yonogi, S., Kumeda, Y., 2011. Comparison of four enrichment broths for the detection of non-O157 Shiga-toxin-producing *Escherichia coli* O91, O103, O111, O119, O121, O145 and O165 from pure culture and food samples. Lett. Appl. Microbiol. 53, 167–73.
- Kanki, M., Seto, K., Sakata, J., Harada, T., Kumeda, Y., 2009. Simultaneous enrichment of shiga toxin-producing *Escherichia coli* O157 and O26 and *Salmonella* in food samples using universal preenrichment broth. J. Food Prot. 72, 2065–70.
- Kaper, J.B., Nataro, J.P., Mobley, H.L.T., 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. 2, 123–140.
- Kaper, J.B., O'Brien, A.D., 2014. Overview and Historical Perspectives. Microbiol. Spectr. 2.
- Karama, M., Gyles, C.L., 2010. Methods for genotyping verotoxin-producing *Escherichia coli*. Zoonoses Public Health 57, 447–462. d
- Karmali, M. a., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., Isaac-Renton, J., Clark, C., Rahn, K., Kaper, J.B., 2003. Association of Genomic O Island 122 of *Escherichia coli* EDL 933 with Verocytotoxin-Producing *Escherichia coli* Seropathotypes That Are Linked to Epidemic and/or Serious Disease. J. Clin. Microbiol. 41, 4930–4940.
- Karmali, M.A., 1989. Infection by Verocytotoxin-Producing *Escherichia coli*. Clin. Microbiol. Rev. 2, 15–38.
- Karmali, M.A., Petric, M., Bielaszewska, M., 1999. Evaluation of a microplate latex agglutination method (Verotox-F assay) for detecting and characterizing verotoxins (Shiga toxins) in *Escherichia coli*. J. Clin. Microbiol. 37, 396–399.
- Karmali, M.A., Steele, B.T., Petric, M., Lim, C., 1983. Syndrome associated with faecal *Escherichia coli* in stools. Lancet 1, 619–620.
- Kase, J. a, Maounounen-Laasri, A., Son, I., Lin, A., Hammack, T.S., 2015. Comparison of eight different agars for the recovery of clinically relevant non-O157 Shiga toxin-producing *Escherichia coli* from baby spinach, cilantro, alfalfa sprouts and raw milk. Food Microbiol. 46, 280–7.
- Kase, J.A., Maounounen-Laasri, A., Son, I., Deer, D.M., Borenstein, S., Prezioso, S., Hammack, T.S., 2012. Comparison of different sample preparation procedures for the detection and isolation of *Escherichia coli* O157: H7 and Non-O157 STECs from leafy greens and cilantro. Food Microbiol. 32, 423–426.
- Kauffman, M.D., LeJeune, J., 2011. European Starlings (*Sturnus vulgaris*) challenged with *Escherichia coli* O157 can carry and transmit the human pathogen to cattle. Lett. Appl. Microbiol. 53, 596–601.

- Kaufmann, M., Zweifel, C., Blanco, M., Blanco, J.E., Blanco, J., Beutin, L., Stephan, R., 2006. *Escherichia coli* O157 and non-O157 Shiga toxin-producing *Escherichia coli* in fecal samples of finished pigs at slaughter in Switzerland. *J. Food Prot.* 69, 260–266.
- Khakhria, R., Duck, D., Lior, H., 1990. Extended phage-typing scheme for *Escherichia coli* O157:H7. *Epidemiol. Infect.* 105, 511–520.
- Konowalchuk, J., Speirs, J.I., Stavric, S., 1977. Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.* 18, 775–779.
- Konowalchuk, J., Speirs, J.I., Stavric, S., 1977. Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.* 18, 775–9.
- Lacher, D.W., Gangiredla, J., Jackson, S.A., Elkins, C.A., Feng, P.C.H., 2014. Novel microarray design for molecular serotyping of shiga toxin-producing *Escherichia coli* strains isolated from fresh produce. *Appl. Environ. Microbiol.* 80, 4677–4682.
- Lainhart, W., Stolfa, G., Koudelka, G.B., 2009. Shiga toxin as a bacterial defense against a eukaryotic predator, *Tetrahymena thermophila*. *J. Bacteriol.* 191, 5116–5122.
- Law, H.T., Guttman, J.A., 2014. Structural, Molecular and Functional Characteristics of Attaching and Effacing Lesions, in: Morabito, S. (Ed.), *Pathogenic Escherichia coli*. pp. 181–200.
- Lawal, D., Burgess, C., McCabe, E., Whyte, P., Duffy, G., 2015. Development of a quantitative real time PCR assay to detect and enumerate *Escherichia coli* O157 and O26 serogroups in bovine recto-anal swabs. *J. Microbiol. Methods* 114, 9–15.
- Lawson, D.M., Stevenson, C.E.M., 2012. Structural and functional dissection of Aminocoumarin antibiotic biosynthesis: A review. *J. Struct. Funct. Genomics* 13, 125–133.
- Lee, S., Cherla, R.P., Caliskan, I., Vernon, L., Tesh, V.L., 2005. Shiga Toxin 1 Induces Apoptosis in the Human Myelogenous Leukemia Cell Line THP-1 by a Caspase-8-Dependent, Tumor Necrosis Factor Receptor-Independent Mechanism Shiga Toxin 1 Induces Apoptosis in the Human Myelogenous Leukemia Cell Line THP-1 by a Caspas. *Infect. Immun.* 73, 5115–5126.
- Leopold, S.R., Magrini, V., Holt, N.J., Shaikh, N., Mardis, E.R., Cagno, J., Ogura, Y., Iguchi, A., Hayashi, T., Mellmann, A., Karch, H., Besser, T.E., Sawyer, S. a, Whittam, T.S., Tarr, P.I., 2009. A precise reconstruction of the emergence and constrained radiations of *Escherichia coli* O157 portrayed by backbone concatenomic analysis. *Proc. Natl. Acad. Sci. U. S. A.* 106, 8713–8718.
- Leyton, D.L., Sevastyanovich, Y.R., Browning, D.F., Rossiter, A.E., Wells, T.J., Fitzpatrick, R.E., Overduin, M., Cunningham, A.F., Henderson, I.R., 2011. Size and conformation limits to secretion of disulfide-bonded loops in autotransporter proteins. *J. Biol. Chem.* 286, 42283–42291.
- Lin, A., Nguyen, L., Lee, T., Clotilde, L.M., Kase, J. a, Son, I., Carter, J.M., Lauzon, C.R., 2011. Rapid O serogroup identification of the ten most clinically relevant STECs by Luminex microbead-based suspension array. *J. Microbiol. Methods* 87, 105–10.
- Lindsey, R.L., Pouseele, H., Chen, J.C., Strockbine, N.A., Carleton, H.A., 2016. Implementation of Whole Genome Sequencing (WGS) for Identification and Characterization of Shiga Toxin-Producing *Escherichia coli* (STEC) in the United States. *Front Microbiol* 7, 766.
- Lorenz, S.C., Son, I., Maounounen-Laasri, A., Lin, A., Fischer, M., Kase, J. a, 2013. Prevalence of Hemolysin Genes and Comparison of *ehxA* Subtype Patterns in Shiga toxin-producing *Escherichia coli* (STEC) and non-STEC Strains from Clinical, Food, and Animal Sources. *Appl. Environ. Microbiol.* 79, 6301–11.
- Luedtke, B.E., Bono, J.L., Bosilevac, J.M., 2014. Evaluation of real time PCR assays for the detection and enumeration of enterohemorrhagic *Escherichia coli* directly from cattle feces. *J. Microbiol. Methods* 105, 72–79.
- Maeda, E., Murakami, K., Etoh, Y., Onozuka, D., Sera, N., Asoshima, N., Honda, M., Narimatsu, H., Iyoda, S., Watahiki, M., Fujimoto, S., 2015. Does sequence type 33 of Shiga toxin-producing *Escherichia coli* O91 cause only mild symptoms? *J. Clin. Microbiol.* 53, 362–364.
- Mahajan, A., Currie, C.G., Mackie, S., Tree, J., Mcateer, S., Mckendrick, I., Mcneilly, T.N., Roe, A., La Ragione, R.M., Woodward, M.J., Gally, D.L., Smith, D.G.E., 2009. An investigation of the expression and adhesin function of H7 flagella in the interaction of *Escherichia coli* O157: H7 with bovine intestinal epithelium. *Cell. Microbiol.* 11, 121–137.
- Mainil, J., 2013. *Escherichia coli* virulence factors. *Vet. Immunol. Immunopathol.* 152, 2–12.
- Mainil, J.G., Daube, G., 2005. Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? *J. Appl. Microbiol.* 98, 1332–44.
- Majowicz, S.E., Scallan, E., Jones-Bitton, A., Sargeant, J.M., Stapleton, J., Angulo, F.J., Yeung, D.H., Kirk, M.D., 2014. Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. *Foodborne Pathog. Dis.* 11, 447–455.
- Malorny, B., Tassios, P.T., Rådström, P., Cook, N., Wagner, M., Hoorfar, J., 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. *Int. J. Food Microbiol.* 83, 39–48.
- March, S.B., Ratnam, S., 1986. Sorbitol-MacConkey Medium for Detection of *Escherichia coli* O157:H7 Associated with Hemorrhagic Colitis. *J. Clin. Microbiol.* 23, 869–72.

- Margot, H., Zwietering, M.H., Joosten, H., O'Mahony, E., Stephan, R., 2015. Evaluation of different buffered peptone water (BPW) based enrichment broths for detection of Gram-negative foodborne pathogens from various food matrices. *Int. J. Food Microbiol.* 214, 109–115.
- Mariani-Kurkdjian, P., Lemaître, C., Bidet, P., Perez, D., Boggini, L., Kwon, T., Bonacorsi, S., 2014. Haemolytic-uraemic syndrome with bacteraemia caused by a new hybrid *Escherichia coli* pathotype. *New Microbes New Infect.* 2, 127–131.
- Martínez-Castillo, A., Muniesa, M., 2014. Implications of free Shiga toxin-converting bacteriophages occurring outside bacteria for the evolution and the detection of Shiga toxin-producing *Escherichia coli*. *Front. Cell. Infect. Microbiol.* 4, 46.
- Martínez-Castillo, A., Quiros, P., Navarro, F., Mir, E., Muniesa, M., 2013. Shiga toxin 2-Encoding bacteriophages in human fecal samples from healthy individuals. *Appl. Environ. Microbiol.* 79, 4862–4868.
- Masana, M.O., D'Astek, B. a, Palladino, P.M., Galli, L., Del Castillo, L.L., Carbonari, C., Leotta, G. a, Vilacoba, E., Irino, K., Rivas, M., 2011. Genotypic characterization of non-O157 Shiga toxin-producing *Escherichia coli* in beef abattoirs of Argentina. *J. Food Prot.* 74, 2008–17.
- Mathusa, E.C., Chen, Y., Enache, E., Hontz, L., 2010. Non-O157 Shiga toxin-producing *Escherichia coli* in foods. *J. Food Prot.* 73, 1721–36.
- Matthews, L., McKendrick, I.J., Ternent, H., Gunn, G.J., Synge, B., Woolhouse, M.E.J., 2006. Super-shedding cattle and the transmission dynamics of *Escherichia coli* O157. *Epidemiol. Infect.* 134, 131–142.
- McDaniel, T.K., Jarvis, K.G., Donnenberg, M.S., Kaper, J.B., 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl. Acad. Sci. U. S. A.* 92, 1664–1668.
- McDonough, M.A., Butters, J.R., 1999. Spontaneous tandem amplification and deletion of the Shiga toxin operon in *Shigella dysenteriae* 1. *Mol. Microbiol.* 34, 1058–1069.
- McGuckin, M.A., Lindén, S.K., Sutton, P., Florin, T.H., 2011. Mucin dynamics and enteric pathogens. *Nat. Rev. Microbiol.* 9, 265–278.
- McWilliams, B.D., Torres, A.G., 2014. Enterohemorrhagic *Escherichia coli* Adhesins. *Microbiol. Spectr.* 2, EHEC00032013. doi:10.1128/microbiolspec.EHEC-0003-2013
- Medina, M.B., Shelver, W.L., Fratafico, P.M., Fortis, L., Tillman, G., Narang, N., Cray, W.C., Esteban, E., Debroy, A., 2012. Latex agglutination assays for detection of non-O157 Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145. *J. Food Prot.* 75, 819–26.
- Mehta, P.K., Raj, A., Singh, N.P., Khuller, G.K., 2014. Detection of potential microbial antigens by immuno-PCR (PCR-amplified immunoassay). *J. Med. Microbiol.* 63, 627–641. doi:10.1099/jmm.0.070318-0
- Molina, P.M., Sanz, M.E., A. Lucchesi, P.M., Padola, N.L., Parma, A.E., 2005. Effects of acidic broth and juices on the growth and survival of verotoxin-producing *Escherichia coli* (VTEC). *Food Microbiol.* 22, 469–473.
- Monaghan, A., Byrne, B., Fanning, S., Sweeney, T., McDowell, D., Bolton, D.J., 2012. Serotypes and virulotypes of non-O157 shiga-toxin producing *Escherichia coli* (STEC) on bovine hides and carcasses. *Food Microbiol.* 32, 223–9.
- Mora, A., Blanco, M., Blanco, J.E., Pilar, M., Dhahi, G., Thomson-carter, F., Miguel, A., Bartolomé, R., Prats, G., Blanco, J., Mora, A., Blanco, M., Blanco, E., Alonso, M.P., Dhahi, G., Thomson-carter, F., Usera, M.A., Bartolome, R., Prats, G., Blanco, J., 2004. Phage Types and Genotypes of Shiga Toxin-Producing *Escherichia coli* O157:H7 Isolates from Humans and Animals in Spain: Identification and Characterization of Two Predominating Phage Types (PT2 and PT9). *J. Clin. Microbiol.* 42, 4007–15.
- Morabito, S., 2015. Shiga Toxin-converting Phages and pathogenic *Escherichia coli*: a world in motion, in: *Escherichia Coli Mucosal Immune System (ECMIS)*. Ghent.
- Morabito, S., Tozzoli, R., Oswald, E., Caprioli, A., 2003. A mosaic pathogenicity island made up of the locus of enterocyte effacement and a pathogenicity island of *Escherichia coli* O157:H7 is frequently present in attaching and effacing *E. coli*. *Infect. Immun.* 71, 3343–3348.
- Muniesa, M., Schmidt, H., 2014. Shiga Toxin-encoding Phages: Multifunctional Gene Ferries, in: *Pathogenic Escherichia Coli Molecular and Cellular Microbiology*. pp. 57–77.
- Munns, K.D., Selinger, L.B., Stanford, K., Guan, L., Callaway, T.R., McAllister, T. a., 2015. Perspectives on Super-Shedding of *Escherichia coli* O157:H7 by Cattle. *Foodborne Pathog. Dis.* 12, 89–103.
- Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11, 142–201.
- Navarro-Garcia, F., 2014. *Escherichia coli* O104:H4 Pathogenesis: an Enteroaggregative *E. coli*/Shiga Toxin-Producing *E. coli* Explosive Cocktail of High Virulence. *Microbiol. Spectr.* 2, 1–19.
- Neely, M.N., Friedman, D.I., 1998. Functional and genetic analysis of regulatory regions of coliphage H-19B: Location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Mol. Microbiol.* 28, 1255–1267.
- Ngom, B., Guo, Y., Wang, X., Bi, D., 2010. Development

- and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: A review. *Anal. Bioanal. Chem.* 397, 1113–1135.
- Niu, Y.D., Xu, McAllister, T.A., Rozema, E.A., Stephens, T.P., Bach, S.J., Johnson, R.P., Stanford, K., 2008. Comparison of fecal versus rectoanal mucosal swab sampling for detecting *Escherichia coli* O157:H7 in experimentally inoculated cattle used in assessing bacteriophage as a mitigation strategy. *J. Food Prot.* 71, 691–8.
- Noll, L.W., Shridhar, P.B., Shi, X., An, B., Cernicchiaro, N., Renter, D.G., Nagaraja, T.G., Bai, J., 2015. A Four-Plex Real-Time PCR Assay, Based on *rfbE*, *stx 1*, *stx 2*, and *eae* Genes, for the Detection and Quantification of Shiga Toxin-Producing *Escherichia coli* O157 in Cattle Feces. *Foodborne Pathog. Dis.* 12, 787–94.
- Norman, K.N., Strockbine, N.A., Bono, J.L., 2012. Association of nucleotide polymorphisms within the o-antigen gene cluster of *Escherichia coli* O26, O45, O103, O111, O121, and O145 with serogroups and genetic subtypes. *Appl. Environ. Microbiol.* 78, 6689–6703.
- Nou, X., Arthur, T.M., Bosilevac, J.M., Brichta-Harhay, D.M., Guerini, M.N., Kalchayanand, N., Koohmaraie, M., 2006. Improvement of immunomagnetic separation for *Escherichia coli* O157:H7 detection by the PickPen magnetic particle separation device. *J. Food Prot.* 69, 2870–2874.
- O'Brien, A.D., LaVeck, G.D., Griffin, D.E., Thompson, M.R., 1980. Characterization of *Shigella dysenteriae* 1 (Shiga) toxin purified by anti-Shiga toxin affinity chromatography. *Infect. Immun.* 30, 170–179.
- O'Brien, A.D., Lively, T.A., Chang, T.W., Gorbach, S.L., 1983. Purification of *Shigella dysenteriae* 1 (Shiga)-like toxin from *Escherichia coli* O157:H7 strain associated with haemorrhagic colitis. *Lancet* september, 573.
- Ogura, Y., Ooka, T., Asadulghani, Terajima, J., Nougayrède, J.-P., Kurokawa, K., Tashiro, K., Tobe, T., Nakayama, K., Kuhara, S., Oswald, E., Watanabe, H., Hayashi, T., 2007. Extensive genomic diversity and selective conservation of virulence-determinants in enterohemorrhagic *Escherichia coli* strains of O157 and non-O157 serotypes. *Genome Biol.* 8, R138.
- Okrend, A.J.G., Rose, B.E., Lattuada, C.P., 1990. Use of 5-bromo-4-chloro-3-indoxyl-B-D-glucuronide in MacConkeysorbitol agar to aid in the isolation of *Escherichia coli* O157:H7 from ground beef. *J. Food Prot.* 53(11), :941–943.
- Orth, D., Grif, K., Dierich, M.P., Würzner, R., 2007. Variability in tellurite resistance and the *ter* gene cluster among Shiga toxin-producing *Escherichia coli* isolated from humans, animals and food. *Res. Microbiol.* 158, 105–111.
- Parma, Y.R., Chacana, P. a, Lucchesi, P.M. a, Rogé, A., Granobles Velandia, C. V, Krüger, A., Parma, a E., Fernández-Miyakawa, M.E., 2012. Detection of Shiga toxin-producing *Escherichia coli* by sandwich enzyme-linked immunosorbent assay using chicken egg yolk IgY antibodies. *Front. Cell. Infect. Microbiol.* 2, 84.
- Paton, A.W., Paton, J.C., 2002. Direct Detection and Characterization of Shiga Toxigenic *Escherichia coli* by Multiplex PCR for *stx1*, *stx2*, *eae*, *ehxA*, and *saa*. *J. Clin. Microbiol.* 40, 271–274.
- Paton, A.W., Paton, J.C., 1998. Detection and Characterization of Shiga Toxigenic *Escherichia coli* by Using Multiplex PCR Assays for *stx 1*, *stx 2*, *eae4*, Enterohemorrhagic *E. coli hlyA*, *rfb* O111, and *rfb* O157. *J. Clin. Microbiol.* 36, 598–602.
- Paton, A.W., Paton, J.C., 1996. *Enterobacter cloacae* producing a Shiga-like toxin II-related cytotoxin associated with a case of hemolytic-uremic syndrome. *J. Clin. Microbiol.* 34, 463–465.
- Paton, A.W., Srimanote, P., Talbot, U.M., Wang, H., Paton, J.C., 2004. A New Family of Potent AB 5 Cytotoxins Produced by Shiga Toxigenic *Escherichia coli*. *J. Exp. Med.* 200, 35–46.
- Paton, A.W., Srimanote, P., Woodrow, M.C., Paton, J.C., 2001. Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect. Immun.* 69, 6999–7009.
- Paton, A.W., Woodrow, M.C., Doyle, R.M., Lanser, J.A., Paton, J.C., 1999. Molecular characterization of a Shiga toxigenic *Escherichia coli* O113:H21 strain lacking *eae* responsible for a cluster of cases of hemolytic- uremic syndrome. *J. Clin. Microbiol.* 37, 3357–3361.
- Perna, N.T., Plunkett, G., Burland, V., Mau, B., Glasner, J.D., Rose, D.J., Mayhew, G.F., Evans, P.S., Gregor, J., Kirkpatrick, H. a, 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157: H7. *Nature* 409, 529–33.
- Perry, J.D., Freydière, A.M., 2007. The application of chromogenic media in clinical microbiology. *J. Appl. Microbiol.* 103, 2046–55.
- Persad, A.K., Lejeune, J.T., 2014. Animal Reservoirs of Shiga Toxin-Producing *Escherichia coli*. *Microbiol. Spectr.* 2, 1–14.
- Piérard, D., De Greve, H., Haesebrouck, F., Mainil, J., 2012. O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: respective role of cattle and humans. *Vet. Res.* 43, 1–12.
- Pinheiro, L.B., Coleman, V. a, Hindson, C.M., Herrmann, J., Hindson, B.J., Bhat, S., Emslie, K.R., 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Anal. Chem.* 84, 1003–11.
- Possé, B., De Zutter, L., Heyndrickx, M., Herman, L., 2008. Novel differential and confirmation

- plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. *FEMS Microbiol. Lett.* 282, 124–31.
- Prager, R., Lang, C., Aurass, P., Fruth, A., Tietze, E., Flieger, A., 2014. Two novel EHEC/EAEC hybrid strains isolated from human infections. *PLoS One* 9.
- Quiros, P., Martinez-Castillo, A., Muniesa, M., 2015. Improving detection of Shiga toxin-producing *Escherichia coli* by molecular methods by reducing the interference of free Shiga toxin-encoding bacteriophages. *Appl. Environ. Microbiol.* 81, 415–421.
- Rivas, M., Chinen, I., Miliwebsky, E., Masana, M., 2014. Risk Factors for Shiga Toxin- Producing *Escherichia coli*- Associated Human Diseases. *Microbiol. Spectr.* 2, 1–14.
- Rivas, M., Miliwebsky, E., Chinen, I., Roldan, C.D., Balbi, L., Garcia, B., Fiorilli, G., Sosa-Estani, S., Kincaid, J., Rangel, J., Griffin, P.M., 2006. Characterization and Epidemiologic Subtyping of Shiga Toxin-Producing *Escherichia coli* Strains Isolated from Hemolytic Uremic Syndrome and Diarrhea Cases in Argentina. *Foodborne Pathog. Dis.* 3, 88–96.
- Rooks, D.J., Yan, Y., McDonald, J.E., Woodward, M.J., McCarthy, A.J., Allison, H.E., 2010. Development and validation of a qPCR-based method for quantifying Shiga toxin-encoding and other lambdoid bacteriophages. *Environ. Microbiol.* 12, 1194–1204.
- Sánchez, S., Llorente, M.T., Echeita, M.A., Herrera-León, S., 2015. Development of Three Multiplex PCR Assays Targeting the 21 Most Clinically Relevant Serogroups Associated with Shiga Toxin-Producing *E. coli* Infection in Humans. *PLoS One* 10, e0117660.
- Savoye, F., Rozand, C., Bouvier, M., Gleizal, A., Thevenot, D., 2011. Optimized enrichment for the detection of *Escherichia coli* O26 in French raw milk cheeses. *Lett. Appl. Microbiol.* 52, 603–609.
- Saxena, T., Kaushik, P., Krishna Mohan, M., 2015. Prevalence of *E. coli* O157:H7 in water sources: an overview on associated diseases, outbreaks and detection methods. *Diagn. Microbiol. Infect. Dis.* 82, 249–264.
- Scheutz, F., 2014. Taxonomy Meets Public Health: The Case of Shiga Toxin- Producing *Escherichia coli*. *Microbiol. Spectr.* 2, 1–15.
- Scheutz, F., Teel, L.D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Celsa, A.R., Sanchez, M., Persson, S., O'Brien, A.D., 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J. Clin. Microbiol.* 50, 2951–2963.
- Schmidt, H., Beutin, L., Karch, H., 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect. Immun.* 63, 1055–1061.
- Schmidt, H., Bielaszewska, M., Karch, H., 1999. Transduction of enteric *Escherichia coli* isolates with a derivative of Shiga toxin 2-encoding bacteriophage 3538 isolated from *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 65, 3855–3861.
- Schüller, S., 2011. Shiga Toxin Interaction with Human Intestinal Epithelium. *Toxin* 3, 626–639.
- Sekiya, K., Ohishi, M., Ogino, T., Tamano, K., Sasakawa, C., Abe, A., 2001. Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11638–11643.
- Serra-Moreno, R., Jofre, J., Muniesa, M., 2008. The CI repressors of Shiga toxin-converting prophages are involved in coinfection of *Escherichia coli* strains, which causes a down regulation in the production of Shiga toxin 2. *J. Bacteriol.* 190, 4722–4735.
- Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R. V., 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J. Food Prot.* 67, 2342–53.
- Smith, D.L., Rooks, D.J., Fogg, P.C., Darby, A.C., Thomson, N.R., McCarthy, A.J., Allison, H.E., 2012. Comparative genomics of Shiga toxin encoding bacteriophages. *BMC Genomics* 13, 311.
- Smith, H.W., Lingwood, M., 1971. The transmissible nature of enterotoxin production in a human enteropathogenic strain of *Escherichia coli*. *J. Med. Microbiol.* 4, 301–305.
- Smith, J.L., Fratamico, P.M., 2012. Effect of stress on non-O157 Shiga toxin-producing *Escherichia coli*. *J. Food Prot.* 75, 2241–50.
- Soriano, J.M., Rico, H., Moltó, J.C., Mañes, J., 2000. Assessment of the microbiological quality and wash treatments of lettuce served in University restaurants. *Int. J. Food Microbiol.* 58, 123–128.
- Stephens, P.J., Joynson, J. a, 1998. Direct inoculation into media containing bile salts and antibiotics is unsuitable for the detection of acid/salt stressed *Escherichia coli* O157:H7. *Lett. Appl. Microbiol.* 27, 147–51.
- Strain, M.C., Lada, S.M., Luong, T., Rought, S.E., Gianella, S., Terry, V.H., Spina, C.A., Woelk, C.H., Richman, D.D., 2013. Highly Precise Measurement of HIV DNA by Droplet Digital PCR. *PLoS One* 8, 1–8.
- Strockbine, N.A., Jackson, M.P., Sung, L.M., Holmes, R.K., O'Brien, A.D., 1988. Cloning and sequencing of the gene for Shiga toxin from *Shigella dysenteriae* type 1. *J. Bacteriol.* 170, 1116–1122.
- Strockbine, N.A., Marques, L.R.M., Newland, J.W., Smith,

- H.W., Holmes, R.K., O'Brien, A.D., 1986. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infect. Immun.* 53, 135–140.
- Sugiyama, K., Inoue, K., Sakazaki, R., 2001. Mitomycin-supplemented washed blood agar for the isolation of shiga toxin-producing *Escherichia coli* other than O157:H7. *Lett. Appl. Microbiol.* 33, 193–195.
- Swinnen, I. a M., Bernaerts, K., Dens, E.J.J., Geeraerd, a H., Van Impe, J.F., 2004. Predictive modelling of the microbial lag phase: a review. *Int. J. Food Microbiol.* 94, 137–59.
- Sze, M.A., Abbasi, M., Hogg, J.C., Sin, D.D., 2014. A comparison between droplet digital and quantitative PCR in the analysis of bacterial 16S load in lung tissue samples from control and COPD GOLD 2. *PLoS One* 9, 1–6.
- Taniuchi, M., Walters, C.C., Gratz, J., Maro, A., Kumburu, H., Serichantalergs, O., Sethabutr, O., Bodhidatta, L., Kibiki, G., Toney, D.M., Berkeley, L., Nataro, J.P., Houpt, E.R., 2012. Development of a multiplex polymerase chain reaction assay for diarrheagenic *Escherichia coli* and *Shigella* spp. and its evaluation on colonies, culture broths, and stool. *Diagn. Microbiol. Infect. Dis.* 73, 121–8.
- Tarr, P.I., Bilge, S.S., Vary, J.C., Jelacic, S., Habeeb, R.L., Ward, T.R., Baylor, M.R., Besser, T.E., 2000. Iha: A novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect. Immun.* 68, 1400–1407.
- Taylor, D.E., Rooker, M., Keelan, M., Ng, L., Martin, I., Perna, N.T., Burland, N.T.V., Blattner, F.R., 2002. Genomic Variability of O Islands Encoding Tellurite Resistance in Enterohemorrhagic *Escherichia coli* O157:H7 Isolates. *J. Bacteriol.* 184, 4690–98.
- Tenaillon, O., Skurnik, D., Picard, B., Denamur, E., 2010. The population genetics of commensal *Escherichia coli*. *Nat. Rev. Microbiol.* 8, 207–17.
- Tenover, F.C., Arbeit, R.D., Goering, R. V, Mickelsen, P. a, Murray, B.E., Persing, D.H., Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33, 2233–2239.
- Teramura, H., Sekiguchi, J.-I., Inoue, K., 2013. A novel Chromogenic Screening Medium for Isolation of Enterohemorrhagic *Escherichia coli*. *Biocontrol Sci.* 18, 111–115.
- Thomas, K.M., McCann, M.S., Collery, M.M., Logan, A., Whyte, P., McDowell, D.A., Duffy, G., 2012. Tracking verocytotoxigenic *Escherichia coli* O157, O26, O111, O103 and O145 in Irish cattle. *Int. J. Food Microbiol.* 153, 288–296.
- Thompson, R.E., Duncan, G., McCord, B.R., 2014. An Investigation of PCR Inhibition Using Plexor-Based Quantitative PCR and Short Tandem Repeat Amplification. *J. Forensic Sci.* 59, 1517–29.
- Tillman, G.E., Wasilenko, J.L., Simmons, M., Lauze, T. a, Minicozzi, J., Oakley, B.B., Narang, N., Fratamico, P., Cray, A.C., 2012. Isolation of Shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from ground beef using modified rainbow agar and post-immunomagnetic separation acid treatment. *J. Food Prot.* 75, 1548–54.
- Tozzoli, R., Caprioli, A., Cappannella, S., Michelacci, V., Marziano, M.L., Morabito, S., 2010. Production of the subtilase AB5 cytotoxin by Shiga toxin-negative *Escherichia coli*. *J. Clin. Microbiol.* 48, 178–183.
- Tozzoli, R., Grande, L., Michelacci, V., Ranieri, P., Maugliani, A., Caprioli, A., Morabito, S., 2014. Shiga toxin-converting phages and the emergence of new pathogenic *Escherichia coli*: a world in motion. *Front. Cell. Infect. Microbiol.* 4, 80.
- Tozzoli, R., Scheutz, F., 2014. Diarrhoeagenic *Escherichia coli* Infections in Humans, in: Morabito, S. (Ed.), *Pathogenic Escherichia Coli Molecular and Cellular Microbiology*. pp. 1–18.
- Trabulsi, L.R., Keller, R., Tardelli Gomes, T.A., 2002. Typical and atypical enteropathogenic *Escherichia coli*. *Emerg. Infect. Dis.* 8, 508–513.
- Tukey, J.W., 1977. Chapter 1, Exploratory Data Analysis.
- Tutenel, a, 2003. Sensitivity of methods for the isolation of *Escherichia coli* O157 from naturally infected bovine faeces. *Vet. Microbiol.* 94, 341–46.
- Tyler, J.S., Friedman, D.I., 2004. Characterization of a eukaryotic-like tyrosine protein kinase expressed by the Shiga toxin-encoding bacteriophage 933W. *J. Bacteriol.* 186, 3472–3479.
- Tzipori, S., Gunzer, F., Donnenberg, M.S., De Montigny, L., Kaper, J.B., Donohue- Rolfe, A., 1995. The role of the *eaeA* gene in diarrhea and neurological complications in a gnotobiotic piglet model of enterohemorrhagic *Escherichia coli* infection. *Infect. Immun.* 63, 3621–3627.
- Tzschoppe, M., Martin, A., Beutin, L., 2012. A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104:H4 strain from ready-to-eat vegetables. *Int. J. Food Microbiol.* 152, 19–30.
- U.S. department of Agriculture, Laboratory QA/QC, D., 2014. Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges, Laboratory Guidebook. Athens, GA.
- Uhlén, B.E., Oscarsson, J., Wai, S.N., 2014. Haemolysins, in: Morabito, S. (Ed.), *Pathogenic Escherichia Coli Molecular and Cellular Microbiology*. pp. 161–180.

- Uyttendaele, M., Jozwik, E., Tutenel, A., Zutter, L.D.E., Uradzinski, J., Pierard, D., 2001. Effect of Acid Resistance of *Escherichia coli* O157:H7 on Efficacy of Buffered Lactic Acid To Decontaminate Chilled Beef Tissue and Effect of Modified Atmosphere Packaging on Survival of *Escherichia coli* O157:H7 on Red Meat. *J. Food Prot.* 64, 1661–66.
- Uyttendaele, M., Vankeirsbilck, S., Debevere, J., 2001. Recovery of heat-stressed *E. coli* O157:H7 from ground beef and survival of *E. coli* O157:H7 in refrigerated and frozen ground beef and in fermented sausage kept at 7°C and 22°C. *Food Microbiol.* 18, 511–519.
- Van Den Beld, M.J.C., Reubsat, F.A.G., 2012. Differentiation between *Shigella*, enteroinvasive *Escherichia coli* (EIEC) and noninvasive *Escherichia coli*. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 899–904.
- Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., Heyndrickx, M., 2013. Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead lettuce seeds, and their subsequent survival and growth on the seedlings. *Int. J. Food Microbiol.* 161, 214–9.
- Venkatesan, M.M., Buysse, J.M., Hartman, A.B., 1991. Sequence variation in two ipaH genes of *Shigella flexneri* 5 and homology to the LRG-like family of proteins. *Mol. Microbiol.* 5, 2435–2445.
- Verhaegen, B., De Reu, K., Heyndrickx, M., De Zutter, L., 2015a. Comparison of Six Chromogenic Agar Media for the Isolation of a Broad Variety of Non-O157 Shiga toxin-Producing *Escherichia coli* (STEC) Serogroups. *Int. J. Environ. Res. Public Health* 12, 6965–6978.
- Verhaegen, B., Reu, K. De, Heyndrickx, M., Van Damme, I., De Zutter, L., 2015b. Growth of Stressed Strains of Four Non-O157 Shiga Toxin-Producing *Escherichia coli* Serogroups in Five Enrichment Broths. *J. Food Prot.* 78, 1960–66.
- Verstraete, K., De Reu, K., Van Weyenberg, S., Piérard, D., De Zutter, L., Herman, L., Robyn, J., Heyndrickx, M., 2013. Genetic characteristics of Shiga toxin-producing *E. coli* O157, O26, O103, O111 and O145 isolates from humans, food, and cattle in Belgium. *Epidemiol. Infect.* 141, 2503–15.
- Verstraete, K., De Zutter, L., Messens, W., Herman, L., Heyndrickx, M., De Reu, K., 2010. Effect of the enrichment time and immunomagnetic separation on the detection of Shiga toxin-producing *Escherichia coli* O26, O103, O111, O145 and sorbitol positive O157 from artificially inoculated cattle faeces. *Vet. Microbiol.* 145, 106–12.
- Verstraete, K., De Zutter, L., Robyn, J., Daube, G., Herman, L., Heyndrickx, M., de Schaetzen, M.-A., De Reu, K., 2012. Validation of a method for simultaneous isolation of Shiga toxin-producing *Escherichia coli* O26, O103, O111, and O145 from minced beef by an international ring-trial. *Foodborne Pathog. Dis.* 9, 412–7.
- Verstraete, K., Robyn, J., Del-Favero, J., De Rijk, P., Joris, M., Herman, L., Heyndrickx, M., De Zutter, L., De Reu, K., 2012. Evaluation of a multiplex-PCR detection in combination with an isolation method for STEC O26, O103, O111, O145 and sorbitol fermenting O157 in food. *Food Microbiol.* 29, 49–55.
- Verstraete, K., Van Coillie, E., Werbrouck, H., Van Weyenberg, S., Herman, L., Del-Favero, J., De Rijk, P., De Zutter, L., Joris, M.-A., Heyndrickx, M., De Reu, K., 2014. A qPCR assay to detect and quantify Shiga toxin-producing *E. coli* (STEC) in cattle and on farms: a potential predictive tool for STEC culture-positive farms. *Toxins (Basel)*. 6, 1201–21.
- Vimont, a, Delignette-Muller, M.-L., Vernozzy-Rozand, C., 2007. Supplementation of enrichment broths by novobiocin for detecting Shiga toxin-producing *Escherichia coli* from food: a controversial use. *Lett. Appl. Microbiol.* 44, 326–31.
- Vimont, A., Montet, M.P., Lazizzera, C., Bavai, C., 2006a. Modeling and Predicting the Simultaneous Growth of *Escherichia coli* O157:H7 and Ground Beef Background Microflora for Various Enrichment Protocols. *Appl. Environ. Microbiol.* 72, 261–68.
- Vimont, A., Vernozzy-Rozand, C., Delignette-Muller, M.-L., 2006b. Isolation of *E. coli* O157:H7 and non-O157 STEC in different matrices: review of the most commonly used enrichment protocols. *Lett. Appl. Microbiol.* 42, 102–8.
- Vincent, A.T., Derome, N., Boyle, B., Culley, A.I., Charette, S.J., 2016. Next-generation sequencing (NGS) in the microbiological world: How to make the most of your money. *J. Microbiol. Methods*. doi:10.1016/j.mimet.2016.02.016
- Waddell, T.E., Coomber, B.L., Gyles, C.L., 1998. Localization of Potential Binding Sites for the Edema Disease Verotoxin (VT2e) in Pigs. *Can. J. Vet. Res.* 62, 81–86.
- Wang, F., Yang, Q., Kase, J. a, Meng, J., Clotilde, L.M., Lin, A., Ge, B., 2013. Current trends in detecting non-O157 Shiga toxin-producing *Escherichia coli* in food. *Foodborne Pathog. Dis.* 10, 665–77.
- Wang, G., Clark, C.G., Rodgers, F.G., 2002. Detection in *Escherichia coli* of the Genes Encoding the Major Virulence Factors , the Genes Defining the O157:H7 Serotype , and Components of the Type 2 Shiga Toxin Family by Multiplex PCR. *J. Clin. Microbiol.* 40, 3613–19.
- Wang, L., Reeves, P.R., 1998. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. *Infect. Immun.* 66, 3545–3551.
- Wang, L., Rothmund, D., Curd, H., Reeves, P.R., 2003.

- Species-Wide Variation in the *Escherichia coli* Flagellin (H-Antigen) gene. *J. Bacteriol.* 185, 2936–2943.
- Wasala, L., Talley, J.L., Desilva, U., Fletcher, J., Wayadande, A., 2013. Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (Diptera: Muscidae). *Phytopathology* 103, 373–80.
- Watarai, S., Inoue, T.K., Kushi, Y., Isogai, E., Yokota, K., Naka, K., Oguma, K., Kodama, H., 2001. Inhibition of Vero Cell Cytotoxic Activity in *Escherichia coli* O157:H7 Lystaes by Globotriaosylcermaide, Gb3, from Bovine Milk. *Biosci. Biotechnol. Biochem.* 65, 414–419.
- Weagant, S.D., Bound, a J., 2001. Evaluation of techniques for enrichment and isolation of *Escherichia coli* O157:H7 from artificially contaminated sprouts. *Int. J. Food Microbiol.* 71, 87–92.
- Weagant, S.D., Jinneman, K.C., Yoshitomi, K.J., Zapata, R., Fedio, W.M., 2011. Optimization and evaluation of a modified enrichment procedure combined with immunomagnetic separation for detection of *E. coli* O157:H7 from artificially contaminated alfalfa sprouts. *Int. J. Food Microbiol.* 149, 209–17.
- Weaganti, S.D., Bryanti, J.L., Jinnemanz, K.G., 1995. An Improved Rapid Technique for Isolation of *Escherichia coli* O157:H7 from Foods. *J. Food Prot.* 58, 7–12.
- Weber, C., Stephan, R., Druggan, P., Joosten, H., Iversen, C., 2009. Improving the enrichment procedure for *Enterobacteriaceae* detection. *Food Microbiol.* 26, 565–572.
- Wells, J.G., Davis, B.R., Wachsmuth, I.K., Riley, L.W., Remis, R.S., Sokolow, R., Morris, G.K., 1983. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J. Clin. Microbiol.* 18, 512–20.
- Wester, A.L., Brandal, L.T., Dahle, U.R., 2013. Extraintestinal pathogenic *Escherichia coli* carrying the Shiga toxin gene *stx2*. *J. Clin. Microbiol.* 51, 4279–4280.
- Wheeler, S.R., Heard, P., Dufour, C., Thevenot-Sergentet, D., Loukiadis, E., Flowers, R.S., McMahon, W., 2015. Detection of Non-O157 Shiga Toxin-Producing *Escherichia coli* in 375 Grams of Beef Trim Enrichments across Multiple Commercial PCR Detection Platforms. *J. Food Prot.* 78, 196–202.
- Whittam, T.S., Bumbaugh, A.C., 2002. Inferences from whole-genome sequences of bacterial pathogens. *Genet. Dev.* 12, 719–725.
- WHO, 2015. WHO estimates of the global burden of foodborne disease.
- Williams, K.J., Ward, M.P., Dhungyel, O., Van Breda, L., 2014. Relative sensitivity of *Escherichia coli* O157 detection from bovine feces and rectoanal mucosal swabs. *J. Food Prot.* 77, 972–976.
- Windham, W.R., Yoon, S.-C., Ladely, S.R., Haley, J. a, Heitschmidt, J.W., Lawrence, K.C., Park, B., Narrang, N., Cray, W.C., 2013. Detection by hyperspectral imaging of shiga toxin-producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 on rainbow agar. *J. Food Prot.* 76, 1129–36.
- Wylie, J.L., Van Caesele, P., Gilmour, M.W., Sitter, D., Guttek, C., Giercke, S., 2013. Evaluation of a new chromogenic agar medium for detection of Shiga toxin-producing *Escherichia coli* (STEC) and relative prevalences of O157 and non-O157 STEC in Manitoba, Canada. *J. Clin. Microbiol.* 51, 466–71.
- Yang, X., Wang, H., 2014. Parthogenic *E. coli* (Introduction), in: Batt, C.A., Tortorello, M.-L. (Eds.), *Encyclopedia of Food Microbiology*. pp. 695–701.
- Yoshitomi, K.J., Jinneman, K.C., Zapata, R., Weagant, S.D., Fedio, W.M., 2012. Detection and isolation of low levels of *E. coli* O157:H7 in cilantro by real-time PCR, immunomagnetic separation, and cultural methods with and without an acid treatment. *J. Food Sci.* 77, M481–9.
- Zelyas, N., Poon, A., Patterson-Fortin, L., Johnson, R.P., Lee, W., Chui, L., 2016. Assessment of commercial chromogenic solid media for the detection of non-O157 Shiga toxin-producing *Escherichia coli* (STEC). *Diagn. Microbiol. Infect. Dis.* in press.
- Zhang, W., Bielaszewska, M., Pulz, M., Becker, K., Friedrich, A.W., Karch, H., Kuczius, T., 2008. New immuno-PCR assay for detection of low concentrations of shiga toxin 2 and its variants. *J. Clin. Microbiol.* 46, 1292–1297.



## Summary

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Shiga toxin-producing *Escherichia coli* (STEC) remains a major foodborne pathogen of concern across the globe. Ruminants, especially cattle, are colonized by STEC and are regarded as the natural reservoir. Although animals infected with STEC normally show no signs of disease, it can be very pathogenic to humans, causing clinical symptoms ranging from mild to severe diarrhea, possibly complicated with hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP). STEC can be transmitted to humans through many different routes, but mainly through consumption of contaminated foods, like raw or undercooked beef, raw milk, fecal contaminated water, fruits and vegetables. On the other hand person-to-person contact, or direct contact with animal feces or an animal reservoir can also be responsible. STEC O157:H7 remains the most common serogroup within the STEC group. The development of a detection and isolation method has therefore been targeted to this serogroup, resulting in the widely accepted International Organization for Standardization (ISO) standard 16654:2001. However, non-O157 STEC strains are being increasingly recognized and reported as important foodborne pathogens worldwide. Because the method for the detection and isolation of *E. coli* O157:H7 fails to detect these serogroups, accurate methods should be formulated in order to detect and isolate non-O157 STEC serogroups in a broad variety of matrices. The current ISO/TS 13136:2012 describes the detection of the five most common STEC serogroups O26, O103, O111, O145 from food, using a highly-sensitive real-time PCR screening in order to increase the chance to find STEC positive samples. However, the follow-up culture-based isolation of these strains remains problematic. Therefore, we aimed to optimize the strategy for the detection and isolation of non-O157 STEC from a broad variety of food matrices. Furthermore, both low-pathogenic and highly pathogenic STEC strains produce Stx and are frequently recovered from food, assessing the pathogenic potential of these strains remains of utmost importance.

The literature review (*chapter 1*) offers a comprehensive overview of the diarrheagenic *E. coli* pathotype classification, including STEC. The different milestones in the history of STEC are described with the ramifications of each event. A brief insight is provided into the prevalence and epidemiology of STEC. Followed by a description of the colonization by STEC and their prominent colonizing factors. The Shiga toxins and Stx-converting bacteriophage are explained in some detail. *Chapter 1* also provides a summary of the different methods usable for the detection of STEC, in addition the main bottlenecks concerning the cultural isolation are emphasized. Finally, typing methods and virulence factors were described which are commonly used to assess the risk posed by the STEC strains recovered from food.

As this PhD research focused on the improvement of the current detection of STEC, the first step was the cultural enrichment. In *chapter 2* a study is presented where five different enrichment media were compared regarding their resuscitation and enrichment capabilities for a number of

non-O157 STEC strains. These five enrichment media included modified Tryptic Soy Broth (mTSB), Buffered Peptone Water (BPW), BPW with sodium pyruvate, Brila (Merck) and Stec Enrichment Broth (SEB; Bio-Rad). In order to investigate these capabilities the STEC cells were exposed to three types of stress (acid, cold and freeze), mimicking conditions often encountered during food processing and storage. The behaviour of the different non-O157 STEC strains was evaluated. Freeze stress caused the most lethal injury, as well as the most sub-lethal injury in the surviving population. While on the other hand, acid stress seemed to have almost no effect on the STEC cells. The growth dynamics of the stressed and non-stressed non-O157 STEC cells in five enrichment media were investigated using total viable counts. To compare growth capacities, the growth after seven and 24 hours enrichment was measured and lag phases and maximum growth rates were also calculated. In general, growth in BPW resulted in a short lag phase followed by a high maximum growth rate during the enrichment of all tested strains using all stress types. Furthermore, BPW ensured the highest STEC count after seven hours of growth. Supplementing the medium with sodium pyruvate did not improve the growth dynamics. The two selective media, Brila and SEB, were less efficient than BPW but Brila's enrichment performance was remarkably better than SEB. This study shows that irrespective of the effect of background flora, BPW is still recommended for resuscitation of stressed non-O157 STEC.

A suitable cultural enrichment step is important for an effective real-time PCR-based detection and cultural isolation of STEC. While, the real-time PCR-based screening has become indispensable for the detection of STEC, the main bottleneck remains the isolation step. However, obtaining an isolate is crucial for the confirmation of the positive results of the PCR screening. In addition it offers the opportunity for further characterization. The lack of clear-cut metabolic and antimicrobial resistance features of most non-O157:H7 STEC strains means no single chromogenic isolation medium has yet been developed that allows cultivation and differentiation of all STEC from food samples. For this purpose six chromogenic agar media were qualitatively and quantitatively evaluated in *chapter 3*. This study included Tryptone Bile X-glucuronide agar (TBX), Rainbow® Agar O157 (RB), Rapid *E. coli* O157:H7 (RE), Modified MacConkey Agar (mMac), CHROMagar™ STEC (Chr ST) and chromID™ EHEC (Chr ID). During this study, 45 *E. coli* strains were used, including 39 STEC strains belonging to 16 different O serogroups and 6 non-STEC *E. coli*. All *E. coli* strains were able to grow on TBX and RB, whereas one STEC strain was unable to grow on Chr ID and a number of other STEC strains did not grow on mMac, CHROMagar STEC and Rapid *E. coli* O157:H7. However, only the latter three agars were selective enough to completely inhibit the growth of the non-STEC *E. coli*. Our conclusion was that paired use of a more selective agar such as CHROMagar STEC together with a less selective agar like TBX or Chr ID might be the best solution for isolating non-O157 STEC from food.

In *chapter 4* several food matrices were artificially contaminated with STEC to evaluate the detection and isolation efficiency using the enrichment media selected in *chapter 2* and the isolation media selected in *chapter 3*. The food samples included ground (minced) beef, cattle carcass swab, lettuce mix and sprouted soy beans. These samples were artificially contaminated with low amounts of STEC (0.25 – 1.40 cfu/g) belonging to the most common non-O157 STEC serogroups (O26, O103, O111, O145). All samples were enriched in parallel in BPW and Brila broth. After enrichment, detection was performed using real-time PCR, and isolation using two chromogenic agar media, CHROMagar™ STEC and ChromID™ EHEC. The implementation of an acid treatment procedure was investigated to enhance the recovery. Therefore, inoculation on the agar media was performed either directly after enrichment or after the use of the acid treatment procedure. Furthermore, the use of this procedure was also tested on naturally contaminated food products, using 150 *stx*-positive samples.

Although the real-time PCR Cycle Threshold (Ct) values were lower after enrichment in Brila broth, no significant differences in recovery were observed between both enrichment broths. Both agar media were equally suitable for the isolation of STEC, although a significantly higher recovery was obtained when using both agar media in parallel. For samples with a Ct value above 25, an acid treatment step prior to isolation ensured a significant improvement in the recovery of STEC due to the reduction in background microbiota. This acid treatment procedure proved especially useful for the isolation of STEC from sprouted soy bean samples. Surprisingly for the naturally contaminated food products, no improved recovery could be observed.

Once a STEC strain is recovered from food an assessment should be made, whether this strain poses a risk for public health or not. In *chapter 5*, we characterized a large collection of STEC isolates from food (n=242) and human patients (n=40) in Belgium, in addition to 46 non-STEC *E. coli* isolates from food. For this purpose, the serotype and pathotype were investigated. Of the 328 isolates, 251 (77%) were AE-STEC, 31 (9%) were STEC, 35 (11%) were aEPEC and 11 (3%) were negative for all tested pathotype specific genes. In addition, the genetic relatedness as determined by the combined data of pulsed-field gel electrophoresis (PFGE) and virulence profile was determined. Besides *stx1*, *stx2* and *eae*, the virulence profile consisted of non-LEE (NLE) genes (OI-122; *ent/espL2*, *nleE* and OI-71; *nleA*, *nleF* and *nleH1-2*) and the plasmid encoded genes (*ehxA*, *katP*, *saa*, *subA*). The NLE genes and the plasmid encoded genes, *ehxA* and *katP* were more often detected in AE-STEC compared to STEC. Moreover, the NLE genes (except *nleF*) were more frequently detected in AE-STEC isolated from human patients compared to those from food origin. The analysis of these virulence profiles combined with the PFGE patterns revealed a number of pulsogroups (80% similarity). We observed that certain pulsogroups contain isolates with a potentially higher pathogenicity potential; these pulsogroups also included the human

isolates and are therefore of public health importance. When in the future strains are isolated from food, their virulence potential could be estimated using the described characterization method and pulsogroups in this study.

In *chapter 6* the novel droplet digital PCR (ddPCR) was presented for the quantification of STEC virulence genes *stx1*, *stx2* and *eae* in cattle feces. ddPCR is an end-point amplification PCR capable of absolute quantification independent from any reference material and is less prone to PCR inhibition than qPCR. After optimizing the protocol described by Verstraete *et al.* (2014) for ddPCR analysis, the features of ddPCR and qPCR using two different mastermixes (EMM: TaqMan® Environmental Master Mix 2.0; UMM: TaqMan® Universal PCR Master Mix) were evaluated. Evaluation of the standard curves and both artificial and natural contaminated cattle fecal samples suggested a very good agreement between qPCR using EMM and ddPCR. The limit of quantification of the assays was shown to be 2.75 – 3.06 log g<sup>-1</sup> in artificially contaminated faeces. The upper quantification limit (<10<sup>5</sup> copies µl<sup>-1</sup>) for the ddPCR was notably lower in comparison to qPCR. Furthermore, no PCR inhibition was recorded for both assays. On the other hand, qPCR using UMM was clearly prone to PCR inhibition. In conclusion, the ddPCR assay showed very similar characteristics to the qPCR when using an appropriate specialized mastermix (e.g. EMM). Especially, the independence from any standardized reference material is still a major improvement. Any future studies on shedding and persistence of STEC in the bovine herds should consider the applications of ddPCR.

Finally in chapter 7, results obtained within the scope of this PhD thesis were discussed. Alternatives regarding detection, isolation and characterization of STEC were proposed. Furthermore, some future prospects are formulated.

In conclusion, this doctoral thesis provides insights in the detection and isolation of STEC strains belonging to non-O157 STEC serogroups. The described results indicate that the recovery of these strains from food can be enhanced, especially from “more difficult” food matrices, such as sprouted seeds. Further, isolates from food and human origin were characterized which revealed more insight in the virulence and the relatedness among STEC strains isolated from food circulating in Belgium. Finally, the features of the novel ddPCR was compared to the well-known qPCR for the quantification of STEC virulence genes in cattle feces. Still further research remains necessary to improve the detection method of STEC and to broaden the understanding concerning the virulence of STEC.

## Samenvatting

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Shiga toxine producerende *E. coli* (STEC) is nog steeds een belangrijke voedseloverdraagbare pathogeen op wereldniveau. Herkauwers en vooral runderen worden gekoloniseerd door STEC en treden op als reservoir van deze pathogeen. Terwijl de mens zelf zelden of nooit ziekteverschijnselen vertoont, kan een STEC-infectie hier milde tot zeer ernstige ziekteverschijnselen veroorzaken, eventueel met complicatie zoals het hemolytisch uremisch syndroom (HUS) of thrombotisch thrombocytopenisch purpura (TTP). STEC kan worden overgedragen op de mens via verschillende routes, maar voornamelijk via de consumptie van besmet voedsel, zoals rauw of onvoldoende gebakken rundsvlees, rauwe melk of fecaal verontreinigd water, fruit en groenten. Daarenboven is de overdracht van mens tot mens en het rechtstreekse contact met dieren of hun mest ook vaak de oorzaak van humane infecties met STEC. Wereldwijd worden de meeste STEC uitbraken nog steeds veroorzaakt door het STEC serotype O157:H7. Efficiënte detectie- en isolatiemethodes zijn ontwikkeld voor de opsporing van deze specifieke pathogeen, wat reeds leidde tot een algemeen aanvaarde International Organization for Standardization (ISO) standaard 16654:2001. In de laatste decennia worden echter steeds vaker niet-O157 STEC-serogroepen gerapporteerd als oorzaak van voedseluitbraken. Aangezien de detectie- en isolatiemethode beschreven voor *E. coli* O157:H7 onvoldoende is voor deze serogroepen, dient een nieuwe methode te worden ontwikkeld. Deze methode hoort de detectie en isolatie van alle niet-O157 STEC-serogroepen mogelijk te maken uit een ruim gamma van voedselmatrices. De huidige ISO/TS 13136:2012 beschrijft de detectie van de vijf meest voorkomende STEC-serogroepen O26, O103, O111, O145 in levensmiddelen, gebruikmakend van een gevoelige real-time PCR screening. De daarop volgende isolatiestap blijft echter problematisch. Dit doctoraatsonderzoek heeft tot doel de detectie- en isolatiestrategie van de niet-O157 STEC stammen uit levensmiddelen te optimaliseren. Terwijl alle geïsoleerde STEC-stammen uit levensmiddelen Stx produceren, zullen sommige weinig en andere hoog pathogeen zijn voor de mens. De bepaling van de pathogeniciteit van de verschillende STEC-stammen is daarom cruciaal.

Het literatuuroverzicht (*hoofdstuk 1*) geeft een duidelijk overzicht van de indeling van de diarreeveroorzakende *E. coli* pathotypes, waaronder STEC. De verschillende mijlpalen in de geschiedenis van STEC worden beschreven, met aandacht voor de gevolgen van elke gebeurtenis. De prevalentie en epidemiologie worden bondig toegelicht. Vervolgens wordt een kort overzicht gegeven van de kolonisatie door STEC en de geassocieerde factoren. De Shiga toxines en Stx-dragende bacteriofagen worden in enkele details omschreven. Verder worden in *hoofdstuk 1* de verschillende detectie- en isolatiemethodes van STEC samengevat en de knelpunten omtrent isolatie benadrukt. Tot slot worden de typeringsmethodes en de hiermee geassocieerde virulentiefactoren beschreven die vaak gebruikt worden voor het onderscheiden van pathogene STEC-stammen.

De focus van dit doctoraatsonderzoek lag bij de optimalisatie van de huidige detectiemethode van STEC, beginnend bij de eerste stap, de aanrijking. In *hoofdstuk 2* wordt een studie voorgesteld waarin vijf aanrijdingsmedia werden vergeleken op basis van resuscitatie- en aanrijdingscapaciteiten. De vijf onderzochte aanrijdingsmedia waren Buffered Peptone Water (BPW), Buffered Peptone Water met Natriumpyruvaat (BPWp), modified Tryptone Soy Broth (mTSB), Briljant groen gal lactose broth (Brila) en Stec Enrichment Broth (SEB). Om de capaciteiten van deze media te testen werden een aantal niet-O157 STEC stammen blootgesteld aan drie stresstypes (zuur-, koude- en vriesstress), die de natuurlijke voedselverwerking en bewaring dienen na te bootsen. De tolerantie van de verschillende niet-O157 STEC stammen ten opzichte van deze verschillende stressoren werd geëvalueerd. Vriesstress veroorzaakte naast de meeste letaal beschadigde cellen, ook de meeste subleetaal beschadigde cellen in de overlevende populatie. Terwijl zuurstress bijna geen effect had op de STEC- cellen. De groeidynamiek van de gestresseerde en niet-gestresseerde STEC-cellen in de vijf aanrijdingsmedia werd bepaald aan de hand van tellingen. Om de groeicapaciteiten te vergelijken werd de groei na zeven en 24 uur aanrijking gemeten en werden de lagfase en maximale groeisnelheid berekend. In het algemeen vertoonden alle onderzochte STEC stammen, na blootstelling aan de verschillende stresstypes, een groei met de kortste lagfase gevolgd door de hoogste groeisnelheid in BPW. Bovendien werd de grootste groei na zeven uur aanrijking waargenomen in BPW. De supplementatie van dit medium met natriumpyruvaat kon de groeidynamiek niet verbeteren. Bovendien waren beide selectieve media, Brila en SEB, minder efficiënt dan BPW en vertoonde Brila duidelijk betere groeicapaciteiten ten opzichte van SEB. Deze studie toonde aan dat, met het effect van een achtergrond microbiota niet in acht genomen, BPW nog steeds aanbevolen is voor de resuscitatie van gestresseerde STEC- cellen.

Een geschikte aanrijingsstap is cruciaal voor een doeltreffende real-time PCR detectie en op cultuur gebaseerde isolatie van STEC. Terwijl de real-time PCR detectie van STEC een onmisbare screeningsmethode is geworden, blijft de finale isolatiestap problematisch. Toch is het verkrijgen van een isolaat cruciaal voor de bevestiging van positieve PCR- resultaten. Daarnaast biedt het de mogelijkheid voor verdere karakterisatie. Tot op heden is er nog geen chromogeen isolatiemedium ontwikkeld dat de cultivatie en differentiatie ondersteunt van alle STEC uit levensmiddelen, wegens het gebrek aan gemeenschappelijke metabole en antimicrobiële resistentiekenmerken. Daarom werden er zes verschillende chromogene agarmedia kwalitatief en kwantitatief geëvalueerd in *hoofdstuk 3*. De volgende isolatiemediën werden onderzocht: Tryptone Bile X-glucuronide agar (TBX), Rainbow® Agar O157 (RB), Rapid *E. coli* O157:H7 (RE), Modified MacConkey Agar (mMac), CHROMagar™ STEC (Chr ST) en chromID™ EHEC (Chr ID). Tijdens deze studie werden 45 *E. coli*-stammen aangewend, waarvan 39 STEC stammen behorend

tot 16 verschillende O-serogroepen en 6 niet-STE $C$  *E. coli*. Alle *E. coli*- stammen waren in staat te groeien op TBX, RB en Chr ID (behalve één stam). Hoewel een aantal STEC-stammen niet konden groeien op mMac, Chr ST en RE, waren enkel deze laatste drie media selectief genoeg voor de volledige inhibitie van de groei van de niet-STE $C$  *E. coli*. Het gecombineerde gebruik van zowel een hoog-selectief, zoals Chr ST, en een laag-selectief isolatiemedium, zoals TBX of Chr ID, bleek voorlopig de beste oplossing te bieden voor de isolatie van non-O157 STEC uit levensmiddelen.

In *hoofdstuk 4* wordt de efficiëntie van de geselecteerde aanrijkingsmedia van *hoofdstuk 2* en isolatiemediën van *hoofdstuk 3* geëvalueerd, gebruikmakend van verschillende kunstmatige gecontamineerde voedselmatrices. Deze voedselmatrices omvatten filet américain, karkasswabs van runderen, slamix en sojascheuten. De stalen werden kunstmatig gecontamineerd met lage aantallen STEC- stammen (0.25 – 1.40 cfu/g), behorend tot de meest voorkomende niet-O157 STEC serogroepen (O26, O103, O111, O145). Alle stalen werden in parallel aangerijkt in BPW en Brila. Na aanrijking werd de detectie uitgevoerd met de real-time PCR en de isolatie gebruikmakend van Chr ST en Chr ID. Het gebruik van een zuurbehandeling om de recovery te verbeteren werd onderzocht. Hiervoor werden de isolatiemediën zowel direct na aanrijking geïnoculeerd als na een zuurbehandeling. Bovendien werd de zuurbehandeling ook uitgetest op natuurlijk gecontamineerde levensmiddelen, gebruikmakend van 150 *stx*-positieve voedingsstalen.

Terwijl de real-time PCR Cycle Threshold (Ct) waarden lager waren na aanrijking in Brila, kon er geen significant verschil in recovery worden waargenomen tussen beide aanrijkingsmedia. Ook beide isolatiemediën bleken even geschikt voor de isolatie van STEC, hoewel een hogere recovery kon worden gerealiseerd door het gecombineerde gebruik van beide media. Voor de stalen met een Ct boven de 25 kon de zuurbehandeling de kans op een succesvolle isolatie van STEC verbeteren, door de achtergrond microbiota te onderdrukken. Deze zuurbehandeling bleek bijzonder effectief voor de isolatie van STEC uit de sojascheutstalen. De zuurbehandeling daarentegen, bracht geen betere recovery teweeg bij de natuurlijk gecontamineerde stalen.

Zodra een STEC-stam wordt geïsoleerd uit een levensmiddel dient het risico van deze stam voor de volksgezondheid te worden ingeschat. In *hoofdstuk 5* werd de karakterisatie van een grote collectie STEC-stammen uitgevoerd. Deze stammen werden geïsoleerd uit voeding (n=242) en mensen (n=40) in België. Daarenboven werden nog 46 niet-STE $C$  *E. coli*-voedingsisolaten gekarakteriseerd. De genetische verwantschappen werden bepaald aan de hand van de pulsed-field gel electrophoresis (PFGE) patronen en virulentie profielen. Naast *stx1*, *stx2* en *eae* bestonden deze virulentie profielen uit niet-LEE (NLE) genen (OI-122; *ent/espL2*, *nleE* en OI-71; *nleA*, *nleF* en *nleH1-2*) en plasmide gecodeerde genen (*ehxA*, *katP*, *saa*, *subA*). De NLE genen en plasmide genen, *ehxA* and *katP*, werden vaker gedetecteerd bij AE-STE $C$ -isolaten in vergelijking

met STEC-isolaten. Bovendien werden deze NLE genen, met uitzondering van *nleF*, vaker gedetecteerd bij humane AE-STEC isolaten ten opzichte van voedingsisolaten. De gecombineerde analyse van de virulentie profielen en PFGE-patronen resulteerde in verschillende pulsogroepen (80% similarity). Sommige pulsogroepen bevatten de isolaten met een hogere potentiële pathogeniciteit, waaronder de humane STEC-isolaten. In de toekomst kan de pathogeniciteit worden ingeschat van een geïsoleerde STEC-stam uit levensmiddelen, gebruikmakend van de beschreven karakterisatiemethoden en pulsogroepen in deze studie.

In *hoofdstuk 6* werd de vernieuwende droplet digital PCR (ddPCR) voorgesteld voor de kwantificatie van de virulentie genen van STEC *stx1*, *stx2* en *eae* in rundmest. ddPCR is een eindpunt-amplificatie PCR in staat absolute kwantificatie uit te voeren onafhankelijk van referentiemateriaal. Overigens zou de ddPCR minder gevoelig zijn voor PCR inhibitie ten opzichte van qPCR. Eenmaal het protocol beschreven door Verstraete *et al.* (2014) voor qPCR was geoptimaliseerd voor ddPCR, werden de kenmerken van deze techniek vergeleken met de qPCR gebruikmakend van twee verschillende mastermixen (EMM: TaqMan® Environmental Master Mix 2.0; UMM: TaqMan® Universal PCR Master Mix). De resultaten van de standaardcurven en zowel kunstmatige als natuurlijk gecontamineerde rundmeststalen vertoonden een zeer goede overeenkomst tussen de analyse uitgevoerd met de ddPCR en de qPCR gebruikmakend van EMM. De kwantificatielimiet van beide technieken lag tussen 2.75 – 3.06 log g<sup>-1</sup> voor de kunstmatige gecontamineerde rundmeststalen. De kwantificatie bovenlimiet van ddPCR daarentegen, was opmerkelijk lager (<10<sup>5</sup> kopijen µl<sup>-1</sup>) in vergelijking met qPCR. Er werd geen PCR inhibitie waargenomen voor beide technieken, met uitzondering van de qPCR gebruikmakend van UMM. Als conclusie konden we stellen dat ddPCR zeer gelijkaardige eigenschappen bezit ten opzichte van de qPCR, indien een meer gespecialiseerde mastermix wordt gebruikt. Het grootste voordeel van de ddPCR blijft het vermogen van absolute kwantificatie zonder beroep te doen op referentiemateriaal. Om deze reden zou het gebruik van de ddPCR kunnen worden overwogen in toekomstig onderzoek omtrent de uitscheiding en persistentie van STEC in de runderpopulatie.

Tot slot werden in *hoofdstuk 7* de verkregen resultaten binnen deze doctoraatsthesis besproken. Alternatieven omtrent de detectie, isolatie en karakterisatie van STEC werden voorgesteld en enkele toekomstperspectieven geformuleerd.

Algemeen heeft het onderzoek in deze thesis bijgedragen tot inzichten in de detectie- en isolatiemethodes van STEC stammen behorend tot niet-O157 STEC serogroepen. De verkregen resultaten toonden aan dat de recovery van deze STEC-stammen uit levensmiddelen kan worden verbeterd, voornamelijk voor de “moeilijkere” voedingsmatrices zoals kiemgroenten. Vervolgens werden stammen van zowel voedings- als humane afkomst gekarakteriseerd, wat meer inzicht bood in de virulentie en de verwantschappen tussen STEC stammen in België. Tot slot werden de eigenschappen van de qPCR vergeleken met de nieuwe ddPCR voor de kwantificatie van de virulentiegenen van STEC in rundermest. Verder onderzoek is nodig om de detectiemethode van STEC verder te verbeteren en anderzijds om de kennis omtrent de virulentie van STEC te verruimen



## Curriculum vitae

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Bavo Verhaegen werd geboren op 12 juni 1988 te Jette. Nadat hij was afgestudeerd aan het Koninklijk Atheneum van Etterbeek (KAE), richting Wetenschappen-Wiskunde, studeerde hij verder aan de Universiteit Gent.

Hij behaalde in 2012 met onderscheiding the diploma van Master in de Diergeneeskunde – optie Herkauwers. Zijn eerste onderzoekservaringen werden opgedaan aan de vakgroep Morfologie van de faculteit Diergeneeskunde in het kader van zijn masterthesis. Geboeid door het wetenschappelijk onderzoek trad hij in oktober 2012 in dienst bij de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid van de Universiteit Gent waar hij startte als bursaal op het IDESTEC project van de cel Contractueel Onderzoek van de FOD Volksgezondheid, Veiligheid van de voedselketen en Leefmilieu. Dit project was in samenwerking met het Instituut voor Landbouw en Visserij Onderzoek (ILVO), Eenheid Technologie en Voeding en de diensten voedselpathogenen en biotech lab van het Wetenschappelijk Instituut Volksgezondheid (WIV). In 2013 stapte hij over naar het ILVO, Eenheid Technologie en Voeding, waar hij, na afloop van het IDESTEC project, zijn doctoraatsonderzoek verder zette.

Bavo Verhaegen is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Hij was actief deelnemer en spreker op meerdere nationale en internationale congressen, symposia en werkgroepen.

## Scientific publications

---

**Verhaegen B.**, Rekecki A., Decostere A., Van den Broeck W. (2012) Knelpunten in aquacultuur: enkele risicovolle aspecten van de vroege levensstadia van de vis. Vlaams Diergeneeskundig Tijdschrift. 81:2, 63-69

Elhadidy M., Elkhatib W. F., Elfadl E. A. A., Verstraete K., Denayer S., Barbau-Piednoir E., De Zutter L., **Verhaegen B.**, De Rauw K., Piérard D., De Reu K., Heyndrickx M. (2015) Genetic diversity of Shiga Toxin-Producing *Escherichia coli* O157:H7 recovered from Human and Food Sources in Belgium. Microbiology. 161:1, 112-9.

Seliwiorstow T., Baré J., **Verhaegen B.**, Uyttendaele M., De Zutter L. (2014) Evaluation of a New Chromogenic Medium for Direct Enumeration of *Campylobacter* in Poultry Meat Samples. Journal of Food Protection 77:12, 2012-2218.

**Verhaegen B.**, De Reu K., Heyndrickx M., Van Damme I., De Zutter L. (2015) Growth of Stressed Strains of Four Non-O157 Shiga Toxin-Producing *Escherichia coli* Serogroups in Five Enrichment Broths. Journal of Food Protection. Journal of Food Protection 78:11, 2012-2218

**Verhaegen B.**, De Reu K., Heyndrickx M., De Zutter L. Comparison of Six Chromogenic Agar Media for the Isolation of a Broad Variety of Non-O157 Shigatoxin-Producing *Escherichia coli* (STEC) Serogroups. International Journal of Environmental Research and Public Health. 12, 6965-6978.

**Verhaegen B.**, Van Damme I., Heyndrickx M., Botteldoorn N., Elhadidy M., Verstraete K., Dierick K., Denayer S., De Zutter L., De Reu K. (2016) Evaluation of detection methods for non-O157 Shiga toxin-producing *Escherichia coli* from food. International Journal of Food Microbiology., 219, 64-70.

**Verhaegen B.**, De Reu K., De Zutter L., Verstraete K., Heyndrickx M., Van Coillie E. (2016) Comparison of Droplet Digital PCR and qPCR for the Quantification of Shiga Toxin-Producing *Escherichia coli* in Bovine Feces. Toxins. 8:157, 1-11.

## Abstracts and proceedings

---

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., Botteldoorn N., De Zutter L. (2013). Enrichment efficiency of cold and freeze stressed O26 and O111 STEC serotypes. Abstract in Proceedings of the 18<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 12 & 13 September 2013, Part Poster Presentation Abstracts, p 147

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2014). Comparison of six agar media for the isolation of a range of non-O157 Shiga toxin-Producing *Escherichia coli* serotypes. Abstract in Proceedings of the 10<sup>th</sup> Conference European Symposium on the Food Safety, Budapest, Hungary, 7 - 9 May 2014, Poster Abstracts.

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2014). Growth of pure cultures of stressed non-O157 Shiga toxin-Producing *Escherichia coli* serotypes in five enrichment broths. Abstract in Proceedings of the 10<sup>th</sup> Conference European Symposium on the Food Safety, Budapest, Hungary, 7 - 9 May 2014, Technical Abstracts.

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2014). Growth of pure cultures of stressed non-O157 Shiga toxin-Producing *Escherichia coli* serotypes in five enrichment broths. Abstract in Proceedings of the 19<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 18 & 19 September 2014, Part Poster Presentation Abstracts, p 152.

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2014). Comparison of six agar media for the isolation of a range of non-O157 Shiga toxin-Producing *Escherichia coli* serotypes. Abstract in Proceedings of the 19<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 18 & 19 September 2014, Part Poster Presentation Abstracts, p 151.

Elhadidy M., Elkhatib W., Elfadi E.A.A., Verstraete K., Denayer S., Barbau-piednoir E., De Zutter L., **Verhaegen B.**, De Rauw K., Pierard D., De Reu K., Heyndrickx M. (2014). Genetic diversity of Shiga toxin-Producing *Escherichia coli* O157:H7 recovered from human and food sources in Belgium. Abstract in Proceedings of the 19<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 18 & 19 September 2014, Part Poster Presentation Abstracts, p 119.

Heyndrickx M., De Reu K., Verstraete K., **Verhaegen B.**, Piérard D., De Zutter L., Elkhatib W.F., Elfadi E.A.A., Denayer S., Barbau-Piednoir E., Elhadidy M. (2015) Molecular risk assessment of Shiga toxin-Producing *E. coli* O157:H7 strains in Belgium. 6<sup>th</sup> Congress of European Microbiologists (FEMS 2015 Congress), Maastricht, the Netherlands, 7 – 11 June 2015

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2015). Comparison of enrichment and isolation media for the isolation of non-O157 Shiga toxin-producing *Escherichia*

*coli*. Abstract in Proceedings of the 2<sup>nd</sup> *E. coli* and Mucosal Immune System (ECMIS) symposium, Ghent, Belgium, 10 - 13 July 2015, Part III: Selected poster presentations p70.

**Verhaegen B.**, De Zutter L., Van Damme I., Heyndrickx M., De Reu K. (2015). Evaluation of detection and isolation methods for non-O157 Shiga toxin-Producing *Escherichia coli* in artificially contaminated minced beef, cattle carcass swabs, lettuce mix and sprouted soy. Abstract in Proceedings of the 2<sup>nd</sup> *E. coli* and Mucosal Immune System (ECMIS) symposium, Ghent, Belgium, 10 - 13 July 2015, Part II: Selected oral presentations, p37.

Seliwiorstow T., Dièvert R., Bichot Y., Baré J., **Verhaegen B.**, Uyttendaele M., De Zutter L. (2015) RAPID'Campylobacter: A Reliable Medium for the Enumeration of Campylobacter in Poultry Meat Samples. Abstract in Proceedings of the IAFP 2015, Portland, Oregon, 25 - 28 July 2015, Poster Abstracts.

**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2015). Comparison of enrichment and isolation media for the isolation of non-O157 Shiga toxin-producing *Escherichia coli*. Abstract in Proceedings of the 9<sup>th</sup> International Symposium On Shiga Toxin (Verocytotoxin) Producing *Escherichia coli* Infections (VTEC 2015), Boston, Massachusetts, 13 - 16 September 2015, Part E: Oral abstracts & poster abstracts p127.

**Verhaegen B.**, De Zutter L., Van Damme I., Heyndrickx M., De Reu K. (2015). Evaluation of detection and isolation methods for non-O157 Shigatoxin-Producing *Escherichia coli* in artificially contaminated minced beef, cattle carcass swabs, lettuce mix and sprouted soy. Abstract in Proceedings of the 9<sup>th</sup> International Symposium On Shiga Toxin (Verocytotoxin) Producing *Escherichia coli* Infections (VTEC 2015), Boston, Massachusetts, 13 - 16 September 2015, Part E: Oral abstracts & poster abstracts p126.

**Verhaegen B.**, Van Coillie E., De Zutter L., Verstraete K., Heyndrickx M., De Reu K. (2015). Comparison of droplet digital PCR and qPCR to quantify Shiga toxin-producing *Escherichia coli* in bovine feces. Abstract in Proceedings of the 9<sup>th</sup> International Symposium On Shiga Toxin (Verocytotoxin) Producing *Escherichia coli* Infections (VTEC 2015), Boston, Massachusetts, 13 - 16 September 2015, Part E: Oral abstracts & poster abstracts p128.

**Verhaegen B.**, De Reu K. , De Zutter L., Verstraete K., Heyndrickx M., Van Coillie E. (2015). Comparison of droplet digital PCR and qPCR to quantify Shiga toxin-producing *Escherichia coli* in bovine feces. Abstract in Proceedings of the 20<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 8 & 9 October 2015, Part Poster Presentation Abstracts, p 128.

**Verhaegen B.**, De Zutter L., Van Damme I., Heyndrickx M., De Reu K. (2015). Evaluation of detection and isolation methods for non-O157 Shiga toxin-Producing *Escherichia coli* in artificially

contaminated minced beef, cattle carcass swabs, lettuce mix and sprouted soy. Abstract in Proceedings of the 20<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 8 & 9 October 2015, Part Poster Presentation Abstracts, p 129.

**Verhaegen B.**, De Zutter L., Heyndrickx M., De Reu K. (2015). Shiga toxin-Producing *Escherichia coli* (STEC) in food: Evolution and analytical problems. Hand-outs in Proceedings of the 20<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 8 & 9 Oktober 2015, Part Full papers of key-note speakers, p 69-73.

### Oral presentations

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**Verhaegen B.**, De Reu K., Van Damme I., Heyndrickx M., De Zutter L. (2014). Growth of pure cultures of stressed non-O157 Shiga toxin-Producing *Escherichia coli* serotypes in five enrichment broths. 10th Conference European Symposium on the Food Safety, Budapest, Hungary, 7 - 9 May 2014.

**Verhaegen B.**, De Reu K., Heyndrickx M., De Zutter L. (2014). Isolation of non-O157 STEC in food, looking for a needle in a haystack? Invited speaker, One Health symposium FOCUS ON STEC – HUSEC, Groningen, the Netherland, 11 December 2014.

**Verhaegen B.**, Van Coillie E., De Zutter L., Heyndrickx M., De Reu K. (2015) Comparison of droplet digital PCR and conventional quantitative PCR to detect and quantification of Shiga toxin-producing *E. coli* in bovine feces. Workshop of National Reference Laboratory (NRL) for Allergens, Merelbeke, Belgium 11 June 2015.

**Verhaegen B.**, De Zutter L., Van Damme I., Heyndrickx M., De Reu K. (2015). Evaluation of detection and isolation methods for non-O157 Shiga toxin-Producing *Escherichia coli* in artificially contaminated minced beef, cattle carcass swabs, lettuce mix and sprouted soy. 2<sup>nd</sup> *E. coli* and Mucosal Immune System (ECMIS) symposium, Ghent, Belgium, 10 - 13 July 2015.

**Verhaegen B.**, De Zutter L., Heyndrickx M., De Reu K. (2015). Shiga toxin-Producing *Escherichia coli* (STEC) in food: Evolution and analytical problems. Invited speaker. 20<sup>th</sup> Conference on Food Microbiology, Brussels, Belgium, 8 & 9 October 2015.

**Verhaegen B.**, Barbau-Piednoir E., De Reu K., De Zutter L., Van Damme I., Denayer S., De Keersmaecker S., Heyndrickx M., (2016). Genetic relatedness and virulence profile of Shiga toxin-Producing *Escherichia coli* (STEC) isolated from food in Belgium. 21<sup>st</sup> Conference on Food Microbiology, Brussels, Belgium, 15 & 16 September 2016.



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# **The End**

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**Bavo Verhaegen**

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